STUDIES ON THE COMPLEMENT SYSTEM IN SYSTEMIC LUPUS ERYTHEMATOSUS

Gunnar Sturfelt

Lund 1984

Digitized by the Internet Archive
in 2026

https://archive.org/details/IA41546725_0038

STUDIES ON THE COMPLEMENT SYSTEM IN SYSTEMIC LUPUS ERYTHEMATOSUS

by
Gunnar Sturfelt

Department of Rheumatology,
University of Lund,
Lund, Sweden

Lund 1984

© Gunnar Sturfelt
ISBN 91-7222-706-0
Printed in Sweden
Infotryck ab, Malmö 1984

To
Birgitta, Markus and Niklas

This thesis is based on the following publications, referred to in the text by Roman numerals.

I Sturfelt G, Sjöholm AG & Svensson B: Complement components C1 activation and disease activity in SLE. Int. Archs. Allergy appl. Immun. 70:12-18, 1983.

II Sjöholm AG & Sturfelt G.: Cleavage of C2 in pathological serum and plasma studied by crossed immunoelectrophoresis. Acta path. microbiol. immunol. scand. Sect C. Accepted for publication

III Sturfelt G & Sjöholm AG: Complement components, C activation and the acute phase response in systemic lupus erythematosus. Int. Archs Allergy appl. Immunol. Accepted for publication.

IV Sturfelt G, Johnson U & Sjöholm AG: Sequential studies of complement activation in systemic lupus erythematosus (SLE). Submitted to Scand J. Rheumatol.

V Truedsson L & Sturfelt G: Human factor D of the alternative pathway: Purification and quantitation by enzyme amplified electroimmunoassay. J Immunol Methods 63:207-214, 1983.

VI Sturfelt G & Truedsson L: Complement factor D levels in normal serum: comparison between immunochemical and functional determinations. Acta path. microbiol. immunol scand. Sect. C. 91: 383-389, 1983.

VII Sturfelt G, Truedsson L, Thysell H & Björck L: Serum level of complement factor D in systemic lupus erythematosus (SLE) - an indicator of glomerular filtration rate. Acta Medica Scand. Accepted for publication.

CONTENTS

ABBREVIATIONS 8

INTRODUCTION 9
Systemic lupus erythematosus (SLE) 9
- Historical aspects 9
- The modern concept of SLE 9
- Immunological aspects 11
The complement system 13
- Nomenclature 14
- The classical pathway 14
- The alternative pathway 15
- The membrane attack complex (MAC) 16
- Control of complement activation 16
- Biological functions of complement 17
- Inherited complement deficiences 18
- Measurement of complement 19
- The inflammatory plasma protein response and synthesis of complement components 20
- Complement in SLE 21

THE PRESENT INVESTIGATION 22

PART ONE: COMPLEMENT ACTIVATION IN SLE 23
Patients 23
Methods 26
Complement components, complement activation and acute phase response 28
- Results 28
- Discussion 33
Sequential studies of complement activation 38
- Results 38
- Discussion 39

PART TWO: COMPLEMENT FACTOR D IN SLE 43
Enzyme amplified electroimmunoassay (EAE) 43
for measuring complement factor D
- Methods, materials and results 43
- Discussion 45
Serum levels of D protein in SLE - relation to 48
disease activity and renal function
- Methods, materials and results 48
- Discussion 49

GENERAL SUMMARY 51

ACKNOWLEDGEMENTS 54

APPENDIX 71
Paper I 73
" II 81
" III 97
" IV 123
" V 151
" VI 159
" VII 167

ABBREVIATIONS

AHG	Aggregated human IgG
C	Complement
CR1	C3b receptor
CRP	C-reactive protein
EAE	Enzyme amplified electroimmunoassay
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol (aminoethylether)tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
GFR	Glomerular filtration rate
HAE	Hereditary angioedema
HIG	Hemolysis in gel
LMW	Low molecular weight
MAC	Membrane attack complex
nDNA	native deoxyribonucleic acid
RNP	Ribonucleoprotein
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
Sm-antigen	Smith antigen

INTRODUCTION

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Historical aspects. (cf. Talbott, 1974; Benedek & Rodnan, 1982) In medieval medical literature the term lupus referred to erythematous ulcerations, particularly those occurring in the face. Lupus (=wolf) was used as a descriptive term for ulcerative lesions of the skin (Bitter, 1974). In the middle of the nineteenth century, to denote the characteristic skin lesion of lupus, the French dermatologist Pierre Cazenave introduced the term "lupus erythemateux", later latinized to lupus erythematosus. Moritz Kaposi has been credited with being the first to recognize the existence of systemic, potentially serious forms of lupus erythematosus. Even as late as the turn of the century, the distinction between lupus and other diseases such as tuberculosis was vague. Osler (1903), without using the term lupus, described two patients with unequivocal SLE, both of whom died of renal failure.

During the decades that followed, important contributions by pathologists made lupus erythematosus a more distinct entity (Libman & Sacks, 1924; Baehr et al., 1935). The concept developed of the disease as an acute, highly lethal, predominantly female, disorder and the term "Systemic lupus erythematosus (SLE)" was introduced (Keil, 1937). The findings of immunologic disorders such as false positive tests for syphilis (Hauck, 1910), and the presence of LE-cells (Hargraves, 1949), in SLE stimulated theories of disturbances of the immunological system in the pathogenesis in the disease (Dameshek, 1958).

The modern concept of SLE. From being originally considered as a rare disease with serious prognosis, the clinical spectrum of

SLE is now much broader and an increasing number of mild forms of the disease are now being recognized. There are several possible reasons for this altered concept of SLE, such as the growing awareness of the disease among clinicians, together with the availability of new diagnostic tests and the use of modern classification criteria for SLE in diagnostic procedures (Cohen et al., 1971; Canoso & Cohen, 1979; Tan et al., 1982).

Apart from the preponderance of females among the sufferers from SLE (Talal, 1982), epidemiological studies show considerable ethnographic differences, with high prevalence and incidence in coloured populations (Fessel, 1974; Meddings & Grennan, 1980). In a study from southern Sweden which has a homogenous caucasian population , performed before the introduction of classification criteria for SLE a prevalence of 1/16667 was found for Malmö and 1/33333 for the rest of Scania (Leonhardt, 1964). The prevalence of SLE in a current epidemiological study from the same area in southern Sweden was 1/2573 (female 1/1543; male 1/8518, Nived et al., to be published), somewhat lower figures than reported from racially mixed populations (Fessel, 1974).

SLE may involve virtually any organ in the body, characteristic inflammatory lesions being found in the vasculature and in serosal membranes (Adu & Cameron, 1982; Brentjens & Andres, 1982). Cutaneous manifestations and joint symptoms are most frequent. The cumulative frequencies of some other prominent manifestations are nephritis 40-53 %, pleurisy 31-52 %, pericarditis 20-38 % , neuropsychiatric symptoms 26-50 % (Estes & Christian, 1971; Dubois et al.,1974; Lee et al.,1977; Grigor et al., 1978). Discrepancies between some studies may be partly due to variation in diagnostic definitions (Christian, 1982).

The prognosis in terms of survival has markedly improved during the last decades. Thus, from a 90 % 2-year mortality before 1940 (Cluxton & Krause, 1943), current series show a 10 year survival of higher than 75% with onset of multisystem disease as starting point (Albert et al., 1979). Besides the recognition of an increasing number of mild SLE cases, improved monitoring and

treatment, for instance the introduction of antibiotics and dialysis, have probably contributed (Dubois et al.,1974; Karsh et al.,1979; Coplon, 1983). The long term effects on prognosis in SLE of corticosteroids and immunosuppressive therapy are still controversial (Albert et al., 1979; Kaplan, 1983; Miller & Steinberg, 1983).

In most modern studies infection, and the involvement of the kidneys and the central nervous system have been found to be the commonest primary causes of death (Klemperer et al., 1941; Harvey et al., 1954; Estes & Christian, 1971; Rosner et al., 1982). Myocardial infarction has been considered as a major cause of late death, (Urowitz et al., 1976). Death from malignancies is probably not more frequent in those with SLE than among the population in general (Rosner et al., 1982).

Immunological aspects. One of the characteristic features of SLE is the multiplicity of immunological abnormalities. Both cellular and humoral disorders are seen. Antibodies against one or more constituents of cell nuclei are found in virtually all patients with the SLE syndrome. In addition antibodies directed towards other auto-antigens such as cytoplasmic constituents, immunoglobulins, neuronal and thyroidal tissue are common. Cross reactivities between certain autoantibodies have been documented (Hannestad & Stollar, 1978; Agnello et al., 1980). The presence of an auto-antibody against the C3 convertase of the classical pathway of complement (C) in some SLE sera has been reported though the biological significance of this finding is not yet clear (Daha et al., 1980). Some autoantibodies occur more often in connection with particular clinical features in SLE - for instance, antibodies to native DNA are more common in patients with lupus glomerulonephritis than in patients without renal involvement (Tron & Bach, 1977) . Auto-antibodies to cardiolipin were recently reported to occur in conjunction with thrombosis and thrombocytopenia in SLE (Harris et al.,1983). Immune complexes are found in increased amounts in the circulation, and are also detected in affected organs, often in conjunction with C components. There is increasing evidence that local formation

as well as deposition in the tissues of circulating C fixing immune complexes are of pathogenetic significance in SLE (Inman, 1982; Mannik, 1982).

The serological abnormalities seen in SLE have been associated with a defect of "self tolerance" (Burnet, 1959), or in more recent literature with decreased T suppressor cell functions (Morimoto et al., 1980). It has been suggested that defects in cell-mediated immunity may be secondary to humoral factors such as anti-lymphocyte antibodies (Hahn, 1975; Alarcon-Segovia, 1982). Humoral factors may also be partly responsible for impaired leukocyte functions in SLE. Thus, granulocyte and monocyte phagocytosis and chemotaxis are decreased in the presence of SLE serum (Brandt, 1967; Svensson, 1980; Sturfelt et al., 1983).

The etiology and pathogenesis of SLE is still obscure. Different etiological factors, both environmental and genetic, seem to interact (Walport et al., 1982) . Sex influence (Talal, 1982) and immunogenetic factors, particularly of C and C receptors (Iida et al., 1982; Fiedler et al., 1983) are in focus of interest. An etiologic role of virus (type C retrovirus) has been proposed but remains unproven, in humans as well as in animals SLE (Pincus, 1982). Much of our present knowledge of the pathogenesis of SLE has been gained from observations made in animal models, particularly mice. Polyclonal B cell hyperactivity and hypergammaglobulinemia have been recorded in all mice strains with lupus syndromes. Increased generation of auto-antibodies, and increased amounts of circulating immune complexes associated with activation and depletion of C, have also been reported in mice (Dixon, 1982), features that are even characteristic of human SLE. While deficiency of the T-cell growth factor interleukin-2 is a regular finding in murine lupus studies in human SLE have given discrepant results (Alocer-Verela & Alarcon-Segovia, 1982; Sibbit et al., 1983).

THE COMPLEMENT SYSTEM

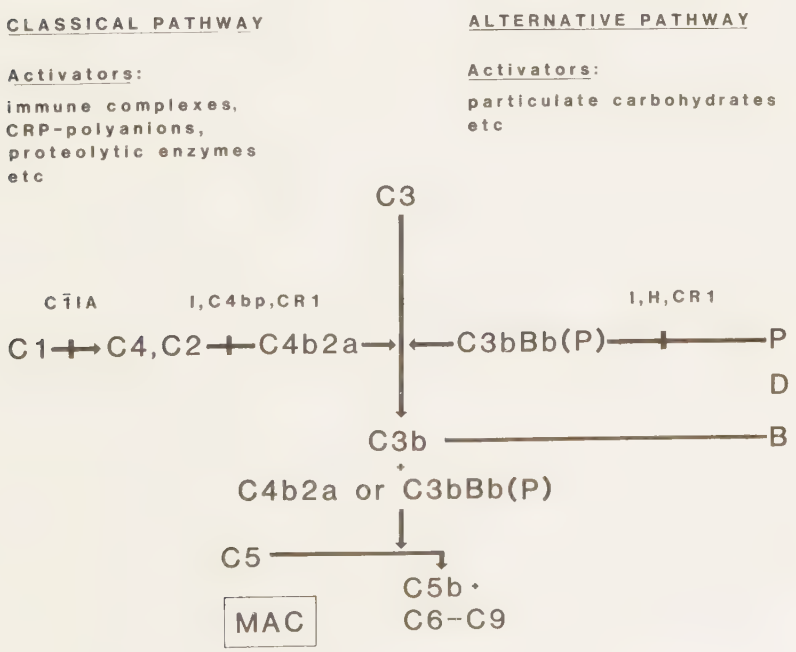
The complement (C) system plays an important role in defense against infection and in the inflammatory reaction. The C system consists of a series of proteins, most of them present in normal serum in an inactive precursor form. Activation of C is accompanied by sequential interactions between C components resulting in the formation of complexes and fragments with important biological functions. Interactions have been recognized between the C system and the clotting, fibrinolytic and bradykinin systems.

The C system can be activated by two main routes (Fig. 1). C1, C2, and C4 are involved in the activation of C3 by the classical

---

Fig 1.

THE COMPLEMENT SYSTEM



pathway, and factors B, D and P together with activated C3 (C3b) constitute the alternative pathway. C5, C6, C7, C8 and C9 form the membrane attack complex (MAC) (Fig 1) which can be recruited by either pathway. C activation is regulated by spontaneous decay of active components and through the action of several control proteins.

Nomenclature. The nomenclature used is that recommended by the World Health Organization (Austen et al., 1970; Alper et al., 1981). Thus, classical pathway components are denoted by arabic numerals and with the exception of C3, the alternative pathway proteins by capital letters. Cleavage products of C proteins are indicated by the addition of a lower case letter - e.g., C2a, C3d. Enzyme activity of activated components or complexes is sometimes designated with a bar over the numeral.

The classical pathway. (cf. Porter & Reid, 1979; Lachmann & Peters, 1982; Loos, 1982; Fearon, 1983). C1, the first component of the classical pathway, consists of three subcomponents C1q, C1s and C1r (Lepow et al., 1963). C1r and C1s are present in serum as a Ca^{2+}-dependent complex (C1r-Ca^{2+}-C1s) fairly loosely associated with C1q (Ziccardi, 1983).

As judged from electron microscopy, C1q consists of six peripheral globular portions connected by radial strands to a central collagen-like common stem (Shelton et al., 1972). The globular portions of C1q contain binding sites for the Fc region of IgG1, IgG2, IgG3 and IgM while the collagen-like stem region provides the binding site for C1r and C1s (Knobel et al., 1974; Reid & Porter, 1976).

Immune complexes containing one IgM molecule, or at least two IgG molecules can bind C1q (Borsos, 1971). It has been suggested that C1q induces an allosteric change in C1r that triggers autocatalytic activation of C1r to an enzyme with specificity

for C1s (Ziccardi, 1983). Activated C1s ($C\bar{1}s$) splits a small fragment, C4a, from C4. The remaining fragment C4b, forms a Mg^{2+} dependent complex with C2. $C\bar{1}s$ then splits C2 to C2a and C2b (Gigli & Austen, 1969). The active enzyme C4b,2a complex is the classical pathway C3 convertase (Kerr, 1980). The active site of this enzyme is situated within C2a (Cooper, 1975; Kerr, 1980) and is capable of cleaving C3 into C3a and C3b (Müller-Eberhard et al., 1967; Bokisch et al., 1969).

In vitro experiments have shown that substances other than immunoglobulins also bind C1q and some of them activate C1. Various polyanionic substances, polyanion-polycation complexes, RNA viruses, certain gramnegative bacteria and C-reactive protein (CRP) complexed with suitable ligands, all have this capacity (Siegel et al., 1974; Siegel et al., 1975; Volonakis, 1983). Proteolytic enzymes can activate C1 (Ratnoff & Naff, 1967; Laurell et al., 1978a), and have also been reported to cleave and activate C3 and C5 (Johnson et al., 1976; Taylor et al., 1977). The in vivo significance of activation by protelytic enzymes is not known, but may be of importance in inflammatory processes.

The alternative pathway. (cf. Müller-Eberhard & Schreiber, 1980; Kazatchkine & Nydegger, 1982) The alternative pathway is activated by polysaccharides and lipopolysaccharides in certain cell membranes without their surfaces first being sensitized by antibodies. The C3 convertase of the alternative pathway is formed by the interaction of C3 and its major fragment, C3b, with the factors B and D. It has been suggested that continuous hydrolysis of an internal thioester in native C3 gives rise to a C3b like molecule that, in the presence of factors B and D and of Mg^{2+}, forms a C3 convertase generating small amounts of C3b by cleavage of C3 (Pangburn & Müller-Eberhard, 1980). If C3b is fixed on activating surfaces, factor B can be bound and a Mg^{2+} dependent complex between C3b and B is formed (Fearon et al., 1973; Vogt et al., 1977; Kazatchkine et al., 1979). In these circumstances, factor B is cleaved by factor D, resulting in release of the Ba fragment and formation of the C3 convertase,

C3b,Bb (Nicholson et al., 1975; Vogt et al., 1977; Lesavre et al., 1979). The low molecular weight protein, factor D (24000 dalton), is present in serum as an active enzyme without known specific inhibitor. The enzymatic activity of factor D is limited to factor B bound to C3b (Lesavre et al., 1979). The dissociation of the Bb subunit of the C3 convertase is retarded by P which binds to C3b forming the stable C3bBbP complex (Fearon & Austen, 1975).

An important part of the alternative pathway is the C3b-dependent, positive feedback mechanism (Müller-Eberhard & Götze, 1972; Fearon et al., 1973). Thus, classical or alternative pathway C3 convertases recruits C3b to form additional C3b,Bb.

The membrane attack complex (MAC). (cf. Müller-Eberhard & Schreiber, 1980). In combination with C3b, the C3 convertases of the classical (C4b,2a) and the alternative (C3b,Bb) pathways acquire C5 convertase activity (Vogt et al., 1978), generating C5a and C5b. A trimolecular complex is built up by the binding of C6 and C7 to surface bound C5b. By incorporation of C8 and C9 with the C5b67 complex the membrane attack complex (MAC) is formed, causing cell lysis (Podack et al., 1980).

Control of complement activation. (cf. Porter & Reid, 1979; Kazatchkine & Nydegger, 1982) The consequences of complement activation are limited by various control mechanisms including spontaneous decay of generated active enzymes and specific control proteins acting at specific points in the activation sequences.

The $C\bar{1}$ inactivator ($C\bar{1}$ IA) inhibits a number of serine proteases in plasma (Ratnoff et al., 1969; Forbes et al., 1970); by binding firmly to $C\bar{1}r$ and $C\bar{1}s$, $C\bar{1}$ IA abolishes their enzymatic activities (Harpel & Cooper, 1975; Ziccardi & Cooper, 1976).

Factor I, in conjunction with its cofactors - C4 binding protein (C4bp) within the classical pathway, and factor H within the alternative pathway - splits both C4b and C3b - i.e., regulates

the activity of the C3 convertases of both the classical and the alternative pathways. Recent studies have suggested that the C3b receptor (CR1) present on certain cell surfaces provides factor I cofactor activity for the cleavage of both C3b (Fearon, 1979) and C4b (Iida & Nussenzweig, 1981). C3b is converted to an inactive protein, C3bi, which is further cleaved to various fragments among which are C3c and C3d/C3dg (α 2D) (Pangburn et al., 1977; Chaplin et al.,1982; Ross,1982). Heterogenous molecular populations, carrying C3d but not C3c antigens, have been identified on C3 activation in serum (Johnson & Holmström, 1982), and in plasma (Teisner et al., 1983); probably this is partly explained by complex formation between C3d/dg and other plasma proteins (Johnson & Holmström, 1982).

The anaphylatoxin inactivator splits the C-terminal amino acid arginine from the fragments C3a, C4a and C5a, whereby their histamine-releasing activity is abolished (see below). However, the "des Arg" derivative of C5a retains both its chemotactic effect and its capacity to aggregate granulocytes.

The S-protein inhibits the membrane attachment of C5b-7 complex, (Podack et al.,1978) and polymerization of C9 within the SC5b-9 complex (Podack et al.,1984).

Biological functions of complement. (cf. Müller-Eberhard & Schreiber, 1980; Fearon & Wong, 1983). The C system has an important protective role in elimination of bacteria, certain viruses and immune complexes. However, the C system also contributes to tissue damage partly owing to to its potential part in eliciting and sustaining inflammatory reaction. During C activation taking place on cell surfaces, MAC is inserted in cell membranes resulting in cytolysis.

Much recent research has been focused on receptors on various cells for split products formed by C activation. Thus, receptors for C3b (CR1) have been demonstrated on erythrocytes, granulocytes, monocytes, B and T lymphocytes, and glomerular podocytes (Ross, 1982). C3b and C4b bound to immune complexes or

particles, facilitate phagocytosis by binding to C3b receptors on phagocytic cells. One function of C3b receptors on erythrocytes may be to facilitate clearance from the blood of immune complexes containing C3b (Ross, 1982). The small peptides C3a, C4a and C5a, are anaphylatoxins that bind to receptors on mast cells and basophils resulting in release of histamine. By binding to receptors on granulocytes and monocytes/ macrophages, C5a also induces both chemotaxis and aggregation of leukocytes. The Bb fragment of factor B induces spreading of macrophages. Cellular receptors for C1q and C3d have been described on various cells, but the importance of the interactions between these C proteins and their receptors remains unclear. Also unknown is the biological significance of the C system for the solubilization of immune complexes (Takahashi et al., 1980), and for the inactivation of viruses (Cooper et al., 1976) - both being C dependent reactions of possible importance in the etiology and pathogenesis of SLE. It has recently been shown that C has influence on the immune response against T cell dependent antigens (Ochs et al., 1983).

Inherited complement deficiencies. (cf. Agnello 1978; Rynes, 1982, Glass et al.,1983) During the last decade, genetically determined deficiencies have been reported for many C components. Deficiency of control proteins leads to increases in C activation and in C factor consumption. Thus hereditary angio-edema (HAE) is due to a deficiency of $C\bar{1}$ IA with secondary deficiencies of C4 and C2, resulting from continuous C1 activation (Donaldson & Evans, 1963; Frank et al.,1976). Isolated genetic deficiencies of components within the classical pathway are associated with inflammatory rheumatic diseases, particularly SLE-like syndromes. SLE-syndromes have also been reported in patients with $C\bar{1}$ IA deficiency and HAE (Donaldsson et al., 1977).

Homozygous deficiency of C1q, C1r, C1s, C2 or C4 clearly predisposes for the development of SLE. C2 deficiency is the most common of these homozygous deficiencies, with a probable frequency of 1/10,000 - 1/20,000 individuals. Clinical SLE-like

syndromes are found in about one-third of C2 deficient individuals. It has also been suggested that heterozygous deficiency of C2 or other classical pathway components is associated with increased susceptibility to SLE (Glass et al., 1976; Fiedler et al., 1983).

Deficiencies of C components within the alternative pathway or MAC are primarily associated with increased susceptibility to bacterial infection (Alper et al., 1972; Petersen et al., 1979).

Recent reports indicate that fewer C3b receptors are expressed on the erythrocytes of SLE patients and their relatives than on those of healthy individuals (Iida et al., 1982).

Measurement of complement. (cf. George & Glass, 1983; Cooper et al., 1983). Some information on the role of C in disease is obtained by examining C concentrations in serum in functional and immunochemical assays. Immune hemolysis can be used to evaluate the integrity of the classical and terminal pathways. Defects in the alternative pathway can be detected by screening assays, using guinea pig or rabbit erythrocytes in the presence of Mg^{2+} and EGTA in hemolytic systems (Truedsson et al., 1981). Individual C components can be measured by functional, usually hemolytic, assays and by immunochemical techniques, using antisera specific for a given complement protein. Transient decreases in concentration of one or several C components are usually caused by C activation.

The measurement of C component cleavage products, or of complexes formed during C activation, makes direct evaluation of C activation possible. Such fragments and complexes can be studied in crossed immunoelectrophoresis and other immunochemical methods. Sensitive enzyme-linked immunosorbent assays (ELISA) have recently been introduced for detecting protein-protein complexes generated during, or as a consequence of, C activation. No methods suitable for studying C metabolism in the acutely ill patient are available at present. The immune

fluorescence technique is commonly used for detecting C components deposited in tissue as a result of C activation.

The inflammatory plasma protein response and synthesis of complement components. (cf. Kushner, 1983) During inflammation a so-called acute phase response appears, as reflected in more or less dramatic increases of certain plasma proteins such as C-reactive protein (CRP), α_1-antichymotrypsin, α_1-antitrypsin and orosomucoid - proteins that are mainly synthesised in the liver. An important triggering mechanism for the acute phase response is probably the monocyte-derived peptide interleukin-1. The acute phase response is modulated by hormones and the various proteins show different kinetic patterns. The concentrations of CRP and α_1-antichymotrypsin increase rapidly in response to surgical trauma (Aronsen et al., 1972).

Concentrations of many C components increase after surgical trauma, indicating that they are involved in the acute phase response (Schutte et al.,1975). Factor B and C1s, in particular, show pronounced acute phase response, while acute phase reactants such as C3 and C4, for instance, are apparently rather slow and moderate in magnitude. Properdin does not react in response to inflammation like an acute phase protein.

C components are mainly synthesized in the liver (Fey & Colten, 1981). In vitro studies have shown that hepatocytes and epithelial cells of the genito-urinary and intestinal tracts and cells of the macrophage/monocyte series synthesize complement components.

CRP is the acute phase protein most widely studied in SLE (Pepys, 1983). While the measurement of CRP has been useful in monitoring disease activity in many rheumatic diseases including rheumatoid arthritis (Hedlund, 1947) its significance in SLE is less clear. In some early studies increased CRP was rarely found in active disease, and marked increases in CRP were taken to suggest superimposed infections (Honig et al., 1977). Subsequently, other investigations indicated that CRP does increase

in SLE, although there are SLE patients with very active disease in whom CRP is not increased at all (Becker et al., 1980). Apart from CRP, hardly any other acute phase proteins have been systematically studied in SLE (Bertouch et al., 1983).

Complement in SLE. (cf. Schur 1982) In one of the earliest complement studies in SLE low CH50 was found in serum from patients with active SLE (Vaughan et al., 1951). Transiently low concentrations of one or more classical pathway components during SLE flares were reported in later studies, and the usefulness of C serum concentrations, reflecting classical pathway activation, as possible indicators of disease activity has been pointed out (Schur, 1975). Evidence has also been given for involvement of the alternative pathway and of regulator proteins of the C system (Ruddy & Austen, 1972; Williams et al., 1974; Wilson et al., 1976; Whaley et al., 1979, Aguado et al., 1980). The relationship between genetically determined C deficiency and increased incidence of SLE syndromes has become apparent (Rynes, 1982).

In synovial, pericardial and pleural fluid from SLE patients with arthritis or serositis, both low concentrations of classical pathway components and conversion products of C3 are found, suggesting immune-complex mediated complement activation (Hunder et al., 1974; Reda & Baigelman, 1980).

Hemolytic C4 concentrations are low in the cerebrospinal fluid of most SLE patients with central nervous system involvement. Normalization of C4 during remission has been reported (Hadler et al., 1973).

The detection of C components in tissue specimens, by means of the immunofluorescence technique, provides further evidence of C activation - particularly when they are deposited together with immunoglobulins and antigens. Biopsies from skin and kidneys are the specimens most commonly studied. In SLE components of the classical pathway are those most often found.

THE PRESENT INVESTIGATION

The aim of this study was to analyze the relationship between C function and clinical manifestations of SLE.

Part one deals with studies of complement activation in relation to clinical manifestations of SLE. For this purpose various methods were used. Components within the classical and alternative pathways were measured both immunochemically and functionally. Activation of C1, C2 and C3 was studied, as reflected in the formation of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ inactivator complexes, C2 cleavage products and circulating C3d. The involvement of classical and alternative pathways was evaluated, and C activation was related to the inflammatory plasma protein response. C activation was analyzed in sequential serum and plasma samples from SLE patients in relation to clinical manifestations.

In the second part of this study, complement factor D in SLE was studied in greater detail, including the development of a simple method for immunochemical measurement of factor D and analysis of D concentrations in serum in relation to inflammatory disease activity and renal function.

PART ONE : COMPLEMENT ACTIVATION IN SLE (I, II,III, IV)

PATIENTS

In 1980, a prospective clinical study of SLE was started at the Department of Rheumatology, Lund. In all patients included in the study, SLE diagnosis was based on the presence of a multisystemic immunological disease in the absence of any other possible diagnosis. Patients with SLE-like syndromes such as discoid lupus or chronic cutaneous vasculitis were excluded as also patients having erosive arthritis with Sjögren's syndrome. Clinical and laboratory investigations were carried out at regular intervals of 6-8 weeks, or as appropriate to the course of the disease. On each occasion a thorough clinical investigation was carried out and serum and EDTA-plasma were collected. In conjunction with each clinical attendance it was decided whether changes in disease activity were present. The evaluation was made without access to results of immunological tests.

The present investigations were designed so as to permit the evaluation of different categories of SLE, based on presence of major or minor manifestations presented during the period of observation (Lightfoot and Hughes, 1976). Thus, patients were assigned to three groups representing mild SLE, severe extra-renal SLE and SLE with glomerulonephritis (III,IV).

By December 31, 1982, 106 patients had been included into the control program. During the observation period twenty-one patients had developed one or more flares of mild SLE, 12 patients had severe extra-renal SLE manifestations and 11 patients had evidence of active lupus glomerulonephritis. For

the studies presented in papers III and IV the first 11 consecutive patients with mild and severe extra-renal SLE, and those with lupus glomerulonephritis were chosen. These 33 patients were followed for 10 - 30 (median 18) months. Four or more of the criteria proposed by the American Rheumatism Association were fulfilled by the 33 patients (Cohen et al., 1971; Tan et al.,1982). Patients with mild SLE and only minor manifestations (usually arthritis in combination with cutaneous manifestations) could be adequately treated with antimalarial drugs and/or low dosages of Prednisolone (<20 mg/day). Other manifestations were considered major and included pleuro-pericarditis, severe symptoms from the central nervous system, renal involvement and miscellaneous visceral manifestations all severe enough to warrant hospitalization. Patients with major manifestations, in the text referred to as severe SLE, with or without renal involvement, were treated with Prednisolone during flares, at a dosage of more than 20 mg/day.

Data on sex distribution and on age and duration of disease on entry to the study are given in the original papers. For detailed data on clinical manifestations and evaluation of activity within different organ systems, the reader is also referred to the original papers.

All patients were ANA positive (I - IV). Precipitating antibodies (Tan & Kunkel, 1966) to nuclear ribonucleoprotein (RNP) and to Sm antigen, were fairly equally prevalent (18 - 27%) among patients with mild SLE as among those with severe SLE (III,IV). Antibodies to native DNA (nDNA) (Aarden et al., 1975) were more often seen in severe than in mild SLE (III,IV). Rheumatoid factors (Waaler-Rose test) were not found in the patients with renal disease (III,IV). A patient with homozygous C2 deficiency with sévere extra-renal manifestations during the study was rheumatoid factor positive, and had positive tests for anti-Sm and anti-RNP, but was anti-nDNA negative (III,IV). One patient with mild SLE manifestations had an IgA deficiency and was rheumatoid factor positive, anti-RNP positive but anti-Sm and anti-nDNA negative.

In a pilot study (I) a group of 8 SLE patients were investigated. All but one of these, who moved away from the retrieval area, joined the SLE control program that followed the pilot study during which clinical evaluation and blood sampling was done at intervals of 2-8 months. C2 cleavage was studied in serum and plasma from some of these SLE patients and in other patients with C activation, which was reported separately (II).

Investigations of complement components, complement activation and C1q binding immune complexes, in serum and plasma, were undertaken at maximal disease activity before treatment was begun, and during quiescent disease at the time of maximal improvement and clinical stability. The first flare was chosen for study when a patient had more than one during the course of the study (I,III). In addition, serial samples of serum and plasma were used for sequential studies both on C1 activation as reflected in the presence of increased amounts of complexes of $C\bar{1}r$ and $C\bar{1}s$ with $C\bar{1}$ inactivator ($C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA), and on activation of C2 and C3 by measuring C2 cleavage and C3d. The results of C activation assays were compared with the concentrations of C1q, C4 and C3 and with C1q binding immune complexes measured by a solid phase assay (IV).

Sampling of serum and plasma. Blood was sampled at all investigations throughout the SLE control program, and venesection was performed at scheduled times. Clotting was allowed to procede for one hour at room temperature (+22 °C) followed by one hour at +4 °C, blood collected in EDTA at 5 mmol/l being treated in the same way. After centrifugation, EDTA-plasma and serum were frozen in aliquots and stored at -80 °C until use. Sera were also collected and handled in the same way at +37 °C until frozen.

METHODS

Complement components

Immunochemical methods. C1q, C1s, C2, C4, C3, C5, factors P and B, $C\bar{1}$ IA (Sjöholm, 1975; Laurell et al.,1978b), as well as factors I, H and C4 bp were measured by electroimmunoassay. Factor D protein was measured by the method described in part two. (I,II,III,IV)

The presence of C1, C1r-C1s and $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA complexes was demonstrated by crossed immunoelectrophoresis (Laurell et al., 1976). $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA complexes were measured by electro-immunoassay (Laurell et al., 1979).(I,III,IV)

C2 cleavage was studied by crossed immunoelectrophoresis (Ganrot, 1972), using barbital buffer 75 mmol/l, pH 8.6, containing EDTA at 2 mmol/l, polyethylene glycol 6000 being added to the agarose gel at 5% w/v in the second step. C2, purified by described methods (Polley & Müller-Eberhard, 1968; Laurell et al., 1976) injected with Freunds adjuvant subcutaneously in a rabbit gave an antiserum monospecific for C2 in immunochemical tests. The IgG fraction of the antiserum was used. The crossed immunoelectrophoresis results were evaluated by planimetry, cleaved C2 being expressed in per cent of total precipitated C2. The lower limit for detection of C2 conversion was 5-10 %, depending on the C2 concentration in the test sample. In a few experiments, C2 was also studied by immunoelectrophoresis (Scheidegger, 1955).(II, III, IV)

C3d, defined as molecules carrying C3d but not C3c antigen, was measured in plasma by "double decker" electroimmunoassay (Brandslund et al., 1981) with anti-C3c and anti-C3d in the gel

as modified by Johnson (1984). Samples were not treated with polyethylene glycol before analysis. C3d values were expressed as percentages of C3d in a normal serum pool treated with inulin (100 mg/ml, 37°C, 120 min) and said to contain C3d at 100 %. The lower limit for detection of C3d was about 6 % of the C3d concentration in the reference preparation.(III,IV)

Bb fragments of factor B were measured in plasma by electroimmunoassay in high electroendosmotic agarose using barbital buffer 75 mmol/l, pH 8.6, with EDTA at 2 mmol/l). Cathodal precipitates were obtained with an antiserum against Bb fragments. Values were given as percentages of the Bb in a normal serum pool treated with inulin (100 mg/ml, 37°C, 5 h).(I)

Functional complement assays. Screen tests for the classical and the alternative pathways were done as described by Truedsson et al., (1981). Functional C2 was determined by hemolytic titration (Ngan, 1977) and by hemolysis in gel (HIG), using C2 deficient serum (Lachmann & Hobart, 1978). Factors B and D were measured essentially as described by Martin et al. (1976). (I,II)

Circulating immune complexes

Circulating immune complexes were measured by a fluid phase C1q binding assay (C1q BA) as described by Zubler et al (1976) and by a solid phase C1q binding assay (C1q SP) essentially according to Scullion (1979).(I,III,IV)

Acute phase proteins

C-reactive protein (CRP), α_1-antitrypsin, α_1-antichymotrypsin and orosomucoid were quantified in electroimmunoassay (Laurell, 1972).(I,III)

COMPLEMENT COMPONENTS, COMPLEMENT ACTIVATION AND ACUTE PHASE RESPONSE IN SLE

Results

Levels of complement components in SLE (I, III). Low concentrations of C1q, C4, C2 or C3 or most often all of them, indicating classical pathway activation, were found in 9/11 patients with active lupus glomerulonephritis (III). The majority of patients with extra renal SLE had normal serum concentrations of C1q, C4, C2 and C3, though the values varied within a wide range; in particular, several patients with mild or severe extra-renal SLE had high C1q concentrations during active disease. Subnormal C1q concentrations were rarely found in mild extra-renal SLE (1/11), but were present in 5/11 patients with severe extra-renal disease. Simultaneously low C1q, C4 and C3 were seen in 2/11 patients with severe, and 1/11 with mild extra-renal SLE during exacerbation (III). C5 concentrations varied essentially within the normal range, often with lower concentrations at remission (I).

One patient was found to have a homozygous C2 deficiency (III,IV). During the observation period, three patients with mild extrarenal SLE and four with lupus glomerulonephritis had persistently low concentrations of one or several components within the classical pathway, most commonly C4 or C2 (III,IV).

C2 was measured both immunochemically and functionally in 8 SLE patients (I). Results obtained by hemolytic titration were similar to those of the hemolysis in gel (HIG) technique. Hemolytic C2 was lower in serum than in plasma samples and C2 functionally measured correlated better with disease activity than did C2 values obtained immunochemically (I).

With few exceptions, factor B concentrations were normal or moderately increased in SLE patients with active disease. There was no significant difference between immunochemical and functional measurements of factor B, nor between its serum and EDTA-plasma values. Factor D serum concentrations were normal or increased (see part two). P varied essentially within the normal range.

Concentrations of $C\bar{1}$ IA were normal or moderately increased, and unrelated to disease activity (I, III); those of of C4 bp were significantly lower in exacerbation than in remission (I). Factors I and H varied essentially within the normal range (I).

Activation of C1, C2, C3 and factor B. The concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA complexes were increased in virtually all patients with active SLE, while measureable amounts of C1r-C1s complexes were detected in 2 only out of 33 patients, both with very low C1q concentrations (I,III). No significant differance of the serum concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA complexes was noted between patients with different SLE manifestations. In quiescent disease, the $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA concentrations were lower than during exacerbation, but still often higher than normal (III). In the pilot study though, this difference was not significant (I). As already mentioned, concentrations of C1 subcomponents varied widely, and for this reason the $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA/C1q quotient was sometimes used as an index of C1 activation. The fact that C1q, C1r and C1s are present at equimolar concentrations in the native macromolecular complex (Ziccardi & Cooper, 1977; Laurell et al., 1978a) might justify such correction of the $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA values.

C3d was detected in 11/11 patients with evidence of active lupus glomerulonephritis, and in 7/11 patients with severe extra renal disease (III). Of patients with mild manifestations, mostly of the skin and joints, only 3/11 had C3d in plasma. Circulating C3d was rarely detected at all during quiescent disease. In paper III, two patients with renal involvement are described who

had C3d in plasma when the disease was clinically inactive, though it is possible that they had subclinical inflammation in the kidneys since progressive impairment of their renal function was noted during the subsequent follow up period. An inverted relationship between C3d and C3 concentrations in plasma was found ($r=-0.54, p<0.01$; Spearman rank correlation test).

C2 cleavage was studied by crossed immunoelectrophoresis, using specific anti C2-antiserum . Normal EDTA-plasma and preparations of hemolytically active C2 gave symmetrical precipitates in the β_2-region. Addition of $C\bar{1}s$ to serum and to EDTA plasma gave rise to a precipitate that was more anodally positioned in the fast β_1-region (II). As judged from studies with immunoelectrophoresis the anodal peak appeared to represent the C2a fragment (Nagasawa & Stroud, 1977). On crossed immunoelectrophoresis fast β_1-peaks were found in plasma samples from the 33 patients with active disease, indicating the presence of circulating C2a (III). C2 cleavage was most pronounced in patients with renal involvement. C2a concentrations were higher in serum than in plasma (II).

An inverted relationship was found between C2a and immunochemical C2 concentrations in SLE patients ($r = -0.66$, $p<0.01$) (III). A close inverted correlation between C2a and hemolytic C2 activity was found in plasma from patients with active SLE and from other patients with C activation ($r = -0.91$, $p<0.01$) (II). C2 cleavage was correlated with C1 activation expressed as a $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$/C1q quotient ($r = 0.59, p<0.01$), and with the C3d/C3 quotient , which was used as an estimate of C3d corrected for circulating C3 ($r=0.63, p<0.01$) (III). Similar correlation was found between $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$/C1q and C3d/C3 quotients ($r = 0.63$, $p<0.01$; Spearman rank correlation test).

Plasma of 8 patients were investigated with regard to factor B activation. One exceptional patient, with signs of disseminated intravascular coagulation, demonstrated factor B conversion with small amounts of Bb fragments in plasma (I).

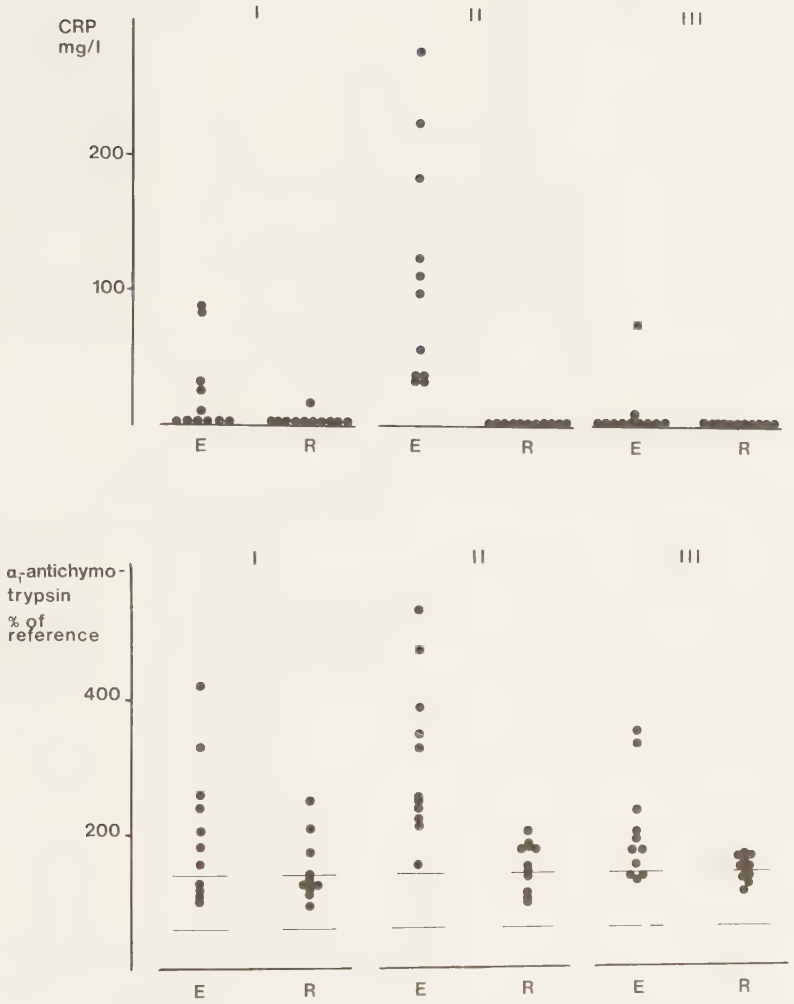


Fig 2.

Concentrations of C-reactive protein (CRP) and of α_1-antichymotrypsin in sera from 33 SLE patients at exacerbation (E) and at remission (R) of disease. I = 11 patients with mild disease, II = 11 patients with severe extra-renal disease, and III = 11 patients with lupus glomerulonephritis. CRP is given in mg/l and α_1-antichymotrypsin in per cent of the normal. Reference areas in serum from normal adults : CRP <12 mg/l; -antichymotrypsin 60 - 140 %.

Inflammatory plasma protein response (I, III). High concentrations of CRP, α_1-antichymotrypsin, orosomucoid and α_1-antitrypsin were found particularly in patients with severe extra-renal manifestations (Fig 2). In these patients CRP concentrations were markedly higher than in patients with mild disease and patients with lupus glomerulonephritis ($p<0.001$; Mann-Whitney U-test). A remarkable finding in some patients with active lupus glomerulonephritis was the absence of measurable CRP even when the concentrations of other acute phase reactants were increased; in particular, one patient with septicemia, during a period of active glomerulonephritis, had very high concentrations of α_1-antichymotrypsin and orosomucoid without concomittant measurable CRP (<12 mg/l) (III). One patient with mild glomerulonephritis and pleuritis had high CRP serum concentrations without evidence of infection (I).

Factor B values correlated with those of α_1-antichymotrypsin during active SLE ($r = 0.51; p<0.01$), while classical pathway components did not show significant correlations with α_1-antichymotrypsin or with any other acute phase protein (Spearman rank correlation test, III).

C1q binding immune complexes (I, III). C1q-binding immune complexes were detected by a solid phase assay in all patients with active renal involvement, and in most patients with extra-renal SLE. In patients with severe disease, the serum concentrations were significantlly higher during exacerbation than during remission ($p<0.01$, Wilcoxon matched-pairs signed-ranks test). Immune complexes, detected by C1q binding fluid phase assay, did not vary with disease activity even in severe SLE (I). Serum samples from two patients - one with skin and joint manifestations, the other with serositis - were repeatedly investigated by solid phase assay without any detectable amounts of immune complexes being found. Since analysis of samples collected at +37°C gave the same results, possible cryoprecipitation during collection procedures could

not explain absence of complexes (unpublished).

Significant correlation was found between immune complexes (measured by C1qSP) and C1 activation expressed as the $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA/C1q quotient ($r=0.52$, $p<0.01$) and also between immune complexes and C2 cleavage (C2a) ($r=0.65, p<0.01$) (III). The correlation between immune complexes and C3d was less marked ($r=0.35, p<0.05$) and no significant correlation was found between immune complexes and the C3d/C3 quotient in patients with presence of C3d in plasma (Spearman rank correlation test) (III).

Discussion

During C activation, limited proteolytic cleavage results in formation of various C fragments. Furthermore, the activation process is characterized by formation of large multimolecular protein complexes. These fragments and complexes have electrophysical and antigenic properties, rendering them distinguishable from the native proteins in immunochemical assay systems (Cooper, 1983). Evidence of classical pathway activation was consistently found in active SLE in the present study, using techniques based on detection of the C cleavage products, C2a, C3d, and protein complexes formed through C1 activation, the $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA complexes. Thus, increased concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA complexes in serum and C2a in plasma could be demonstrated in the patients. C3d, indicating C3 activation, was present in plasma with severe forms of SLE, and active lupus glomerulonephritis in particular was characterized by increased amounts of C3d in plasma. C1, C2 and C3 activation were more pronounced in exacerbation than in quiescence. In patients with extra-renal manifestations, C3d was not present in detectable amounts during remission.

C component concentrations in body fluids reflect the net effects of synthesis and breakdown, which complicates the interpretation of C measurement. However, transient reduction of

C component concentrations strongly suggests C activation. In the present study, hypocomplementemia with low concentrations of C1q, C4 and C3 was found predominantly in patients with significant active glomerulonephritis. In spite of pronounced C activation (as reflected in increased concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA complexes and C2a), concentrations of C components were often normal or even increased in most patients with severe extra-renal SLE. This might be explained by the pronounced inflammatory plasma protein response (including C components) probably preventing development of manifest hypocomplementemia. The reason for the high C1q levels found in some patients during active disease is not evident. It is possible that the high C1q concentrations could be related to C1 activation with dissociation of free C1q (Laurell et al.,1976; Laurell et al.,1978a).

An inverse relationship was found both between the proportion of cleaved C2 and the serum concentrations of C2, and between C3d (as an estimate of C3 cleavage) and the C3 concentrations in serum . The findings suggest increased catabolism and , perhaps, decreased synthesis of C2 and C3 in active SLE. However, the methods used permitted no accurate analysis of the C component concentrations with regard to the relative effects of synthesis and breakdown. Metabolic investigations of the C system require test precedures too cumbersome to be performed in the acutely ill patient, and that cannot be done repeatedly. Some metabolic studies of SLE patients have demonstrated hypercatabolism as a characteristic feature of active SLE . Thus, findings providing evidence that hypocomplementemia in lupus glomerulonephritis is very often caused by hypercatabolism not being offset by increased synthesis were reported by Petz et al. (1977). Hyposynthesis of complement in SLE patients is sometimes found in late stage nephritis, and occasionally in patients with pronounced hypocomplementemia (Petz et al., 1977). It has been suggested, but remains unproven, that circulating C3d sometimes inhibit C3 synthesis (Charlesworth et al.,1974). Furthermore, in vitro studies have shown that C fragments in fluid phase or bound to immune complexes inhibit the synthesis of C2 and factor

B by monocytes (Whaley et al., 1983).

Increased amounts of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes in serum have been demonstrated in rheumatoid arthritis (RA)(Berglund et al.,1980). Despite evidence of C1 activation, none of the RA patients were clearly hypocomplementemic, probably a reflection of the often intense acute phase response in active RA. Nonetheless, a highly significant correlation was found between the concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes and the clinical disease activity of the RA patients.

The present study indicates that measurement of CRP as the only variable is insufficient to assess the acute phase response in SLE. Thus, particularly among patients with lupus glomerulonephritis, normal CRP was often seen despite increased concentrations of other acute phase proteins. A pronounced acute phase response, also of CRP, was observed in many patients with severe extrarenal SLE. Thus, measurement of CRP and of C1q, C4, C3 and circulating C3d made possible some degree of distinction between clinical categories of SLE.

The functional assay for C2 gave results that were more closely correlated with disease activity in the SLE patients than those obtained with the electroimmunoassay (I). Studies using crossed immunoelectrophoresis made it clear that this discrepancy was dependent on the antiserum used in the electroimmunoassay which detected both native and cleaved C2. Thus, a close inverse correlation ($r= -0.91$) was found between C2a and C2 hemolytic activity in serum and plasma samples from patients with SLE and other diseases with C activation (II). Circulating C2a is present in SLE, particularly in active disease, as indicated by the demonstration of C2a in EDTA plasma (III). The higher C2a concentration in serum than in plasma was probably due to the occurrence of C activation after sampling (II). In a recent report, Ueda et al. (1983), found antigenically intact but hemolytically inactive C2 in sera from patients with active SLE, but closely correlated functional and immunochemical C2 values in inactive SLE, which is in agreement with our results.

In contrast to the case of C2, measurement of factor B gave similar results with both functional and immunochemical methods, which argues against appreciable cleavage of factor B in SLE. This was supported by the normal or high factor B concentrations during active disease, and the absence of detectable amounts of Bb fragments in most patients with active SLE (I, III). Factor B was the only C component that significantly correlated with acute phase reactants such as α_1-antichymotrypsin.

These findings are in agreement with a recent report by Mayes et al. (1983), where, using a sensitive ELISA assay, no evidence was found of activation of the alternative pathway in the circulation in SLE . Furthermore, the capacity of serum from patients with active SLE to generate chemotactic activity on zymosan treatment is essentially normal (Sturfelt et al., 1983). However, other investigators have found evidence of increased catabolism of factor B in active SLE (Wilson et al.,1976; Aguado et al.,1980), and deposits of alternative pathway components are sometimes found in kidney biopsies from patients with lupus glomerulonephritis (Westberg et al., 1971). Although limited activation of the alternative pathway may occur through C3b feedback and through tissue bound immune complexes, the present study does not support the idea that the alternative pathway plays a critical role in SLE .

C1q binding immune complexes were more closely correlated with C1 activation ($C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$/C1q) and with C2 cleavage (C2a) than with C3 cleavage (C3d). The reason for this is unknown but perhaps circulating immune complexes play a predominant role in the activation of C1 and C2. Circulating C3d might more clearly reflect activation caused by tissue bound immune complexes. Metabolic studies provide some evidence that the presence of circulating C3d is associated with extra-vascular C3 activation (Charlesworth et al., 1974).

There are strong indications that the C system through classical pathway activation (the characteristic feature of active SLE in

the present study), is involved in the pathogenesis of SLE (Inman, 1982). On the other hand , apparently somewhat paradoxically, there is a marked correlation between disposition to develop SLE and deficiencies of C components and C receptors. Mechanisms of possible pathogenetic significance in SLE, partly explaining this paradox, are the importance of C components and C receptors for solubilization and clearance of immune complexes (Takahashi et al., 1980), and perhaps even for the neutralization and killing of viruses (Cooper et al., 1976).

SEQUENTIAL STUDIES OF COMPLEMENT ACTIVATION (IV)

Results

33 SLE patients were followed for 10 - 30 months with repeated clinical examinations and blood sampling at regular intervals of 6 - 8 weeks. Examinations and blood sampling were also done at the first sign of clinical disease activity, at points of maximal disease activity prior to start of treatment, or as appropriate to the course of disease. Changes in laboratory variables that occurred during intervals before and after flares were analyzed.

The concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes increased in most patients already two months before flare. The changes of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ concentrations from four to two months prior to flare were significant in patients with mild disease, and when all 33 patients were taken as a whole. C2a concentrations in plasma studied in 6 patients, two in each group, showed alterations prior to flares very similar to those of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes. C3d in plasma was consistently present from the onset of lupus glomerulonephritis exacerbations and mostly (in 7/11 cases) in severe extrarenal SLE. Only in three patients with mild SLE, however, could C3d be demonstrated in detectable amounts. The levels of C1q binding immune complexes did not increase significantly prior to flare.

Clinical remission was accompanied by decreasing concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes and C3d, and of C1q binding immune complexes in severe SLE. Although C3d was rarely detected in plasma during remission, plasma C3d was occasionally increased during periods of low active disease in patients with significant glomerulonephritis. In three patients that

subsequently developed renal failure, plasma C3d was invariably present from the onset of the observed flare throughout the study. Persistence of circulating C3d was not consistently associated with low C3 concentrations.

During periods of quiescent disease exceeding 6 months (>3 control intervals) the serum concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes were stable. $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ concentrations in all patients were compared in samples obtained at the start and at the end of a 2 month period during such prolonged quiescence. The differences varied between -8% and +6% (median 0%) of the reference (unpublished).

The serum concentrations of C1q decreased significantly during exacerbation in patients with renal involvement but otherwise the alterations of C components were not significant during the intervals studied prior to and after flares. Five patients with mild SLE, one with severe extra-renal SLE, and four with lupus glomerulonephritis, had 2 clinically distinct flares during the observation period. The frequency of persisting hypocomplementemia was not increased among the patients with several flares during the observation time.

Discussion

Hitherto, in the absence of known etiology, treatment of SLE has been symptomatic and guided by the extent of organ involvement and degree of disease activity, the clinical assessment of these variables still being the physician's safest guide in the choice of therapy (Hughes, 1982). Exacerbations of SLE are regularly accompanied by various immunological aberrations, some of which are considered useful in monitoring the disease. Decreasing concentrations of C4 (Davis et al.,1977), and raised concentrations of C1q binding immune complexes (Abrass et al.,1980) and of antibodies to native DNA (nDNA)(Swaak et al.,1979), have been reported to precede recrudescence of SLE. However, assays for anti-nDNA antibodies, and also perhaps the

C1q binding immune complexes, suffer from the fact that not all patients with the SLE syndrome have positive tests during active disease.

Since indicators of activation of the classical pathway, $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes, C2 cleavage (C2a) and C3 fragmentation (C3d) were shown to vary with disease activity (III), sequential measurement of these components were performed to analyze their possible value in monitoring SLE.

Augmented C1 activation preceded exacerbation by several weeks in most SLE patients, particularly in extra-renal disease. In lupus glomerulonephritis analysis was sometimes complicated by the appearance of minor extra-renal manifestations during periods of low active renal disease. Furthermore, it has been shown that exacerbations develop more rapidly in lupus glomerulonephritis than in other forms of lupus (Lloyd & Schur, 1980).

C2 activation serially studied in a few patients from the various SLE categories gave results that agreed with those of C1 activation. C3d fragments were found in severe SLE but rarely in conjunction with minor SLE manifestations. The presence of C3d was particularly informative in lupus glomerulonephritis, since it was invariably found from onset of active disease but rarely during inactive disease. At variance with the present investigations, Morrow et al., (1983) found no association between C3d concentrations in plasma and clinical disease activity in SLE. The design of Morrow's study, in which the patients were not classified according to principal features such as renal involvement, might partly account for this discrepancy. More in line with our results, Perrin et al., (1975) found increased amounts of plasma C3d in SLE patients, particularly in severe disease with renal involvement.

The prospective sequential studies suggested that persistent C3d was a sign of progressive renal disease. Thus, three patients that had virtually persistent C3d from the onset of flare

throughout the observation period, in spite of initial clinical stability, subsequently developed progressive renal failure. On the other hand, clinical improvement was accompanied by decreasing concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes and of C3d when present in severe SLE.

Of the C components studied, C1q concentrations regularly decreased during the early phase of exacerbation while C4 and C3 varied without significant relation to trends in disease activity. In the patients who had persistently low concentrations of one or more classical pathway components during the observation period, genetically determined partial defects could not be excluded, such defects having been reported to be quite common in SLE (Fiedler et al., 1983). Somewhat in contrast to the findings of Jarret et al., (1981) no significant association between persistent hypocomplementemia and progressive disease was noted in the present study.

A few years ago , circulating immune complexes, measured by C1q solid phase assay, were shown to provide predictive information of disease activity in SLE (Abrass, 1980). The present investigations showed a relationship to exist between clinical improvement and decrease in the amounts of C1q binding substances, but did not confirm that the C1q solid phase assay could predict SLE flare.

No convincing evidence that corticosteroids or cytostatic drugs *per se* significantly influence C activation and synthesis in SLE patients was found by Petz et al. (1977), even if difficulties are associated with such interpretations in the ill patient. Thus, the mechanism by which treatment reduced the concentrations of C activation products in the patients is not known but might be related to influence on C activating immune complexes. In six patients with renal involvement plasma exchange was carried out for short periods (1 - 2 weeks) as an adjunct to immunosuppression. It should be pointed out, however, that no samples obtained within a week of such treatment were used in the present investigation.

In conclusion, the present study shows that serial measurement of variables of classical pathway activation might be useful in monitoring SLE. Tests for C activation were more informative than measurement of C components and of C1q binding immune complexes. However, C component concentrations are often valuable in establishing diagnosis of SLE and to strengthen a clinical impression of disease activity (Lightfoot & Hughes, 1976).

The study of other C fragments than dealt with here, for instance of C4, might provide additional information. The findings in the present study should encourage development of new techniques for measuring C fragments and protein complexes formed by C activation. The question of whether increased accuracy of the information gained from assays for C activation could be obtained by combination with assays for auto-antibodies (anti-nDNA) and possibly circulating immune complexes warrants further study.

PART TWO : COMPLEMENT FACTOR D IN SLE

Using a HIG assay, (Martin et al., 1976) high factor D activity was observed in serum and in plasma of 8 SLE patients (I). No relationship between factor D concentrations and disease activity was found. The highest factor D concentrations were present in patients with reduced glomerular filtration rate, but there was no clear relationship between renal function and D hemolytic activity. The findings warranted further studies. Two main objects were: 1. Development of an immunochemical method for measuring of D protein, and 2. Analysis of the influence of the inflammatory process and renal function on factor D serum concentrations.

ENZYME AMPLIFIED ELECTROIMMUNOASSAY (EAE) FOR MEASURING COMPLEMENT FACTOR D

Methods, materials and results

Purification of factor D. Factor D was purified by combination of ion-exchange chromatography and gel filtration essentially as described by Volanakis et al., (1977). The yield of isolated protein in the purification process was approximately 30 %. Analysis on SDS-polyacrylamide gel electrophoresis revealed only one band at a molecular weight of approximately 25000 daltons. With the isolated protein a rabbit antiserum was raised that without previous absorption was monospecific for factor D. (V)

Production of anti-factor D antiserum. Rabbit antiserum against human complement factor D was produced. The specificity of the antiserum was tested with double immunodiffusion, whereby unabsorbed anti-D antiserum gave a single precipitation line against purified factor D and D in normal human serum with an identity reaction. Furthermore, the anti-D antiserum inhibited the D hemolytic activity of normal human serum in the HIG assay for factor D. (V)

Development of enzyme amplified electroimmunoassay (EAE) for factor D. An electroimmunoassay was developed in which an enzyme amplifying technique was used to stain immunoprecipitates (Löfberg & Grubb, 1979). Thus, horseradish peroxidase-conjugated swine antibodies against rabbit immunoglobulins were applied after electrophoresis. Immunoprecipitates were visualized by adding the perioxidase substrate 3 amino-9-ethylcarbazole. (V)

The precipitates appearing in the electroimmunoassay were of optimal height when low endosmotic agarose was used. Trials with different buffers revealed anodal peaks the in presence of EDTA, but with Ca^{2+} in the buffer both anodal and cathodal precipitates were obtained. For this reason EDTA was used in the electrophoresis buffer. (V)

Addition of polyethylene glycol 6000 to the gel made the precipitates distinct. Lower precipitates were obtained with purified D and pathological sera containing high D concentrations with dilution in buffer compared with dilution in D depleted serum (VI)

The lower limit for detection of factor D by EAE was about 50 µg/l i.e. 3 % of normal.

Measurement of factor D in normal human serum. Comparison between immunochemical and functional determinations. In a reference serum pool (148 normal blood donors), the concentration of D protein was 1.6 mg/l as measured by EAE (V). Sera were

studied from 144 healthy blood donors, 112 men and 32 women, aged 19-61 (median 40 years). The 95 % geometric confidence area for factor D was 75 - 143 % as measured by EAE, and 65-171 % by an HIG assay for factor D (Martin et al.,1976). In sera from 6 elderly subjects (aged 60-80) normal D values were obtained. The difference between the mean D values in men and women was not significant when measured by EAE. Sera from 29 healthy children, aged 2-10 years (median 6 years), showed lower D values than adult sera. The 95 % geometric confidence areas were 56-95 % by EAE and 41-112 by HIG assay in children.(VI)

Further studies were performed with the HIG assay and with a tube assay described by Lesavre et al., (1979) for comparison of functional and immunochemical measurement of factor D. A tendency of the functional assays to give higher values than the immunochemical method in the high range and, conversely, lower D values than the EAE within the low range was not statistically significant. Functional (HIG assay) and immunochemical values in the 144 healthy adults were correlated ($r = 0.52, p<0.001$). Duplicate measurements of 20 sera showed a variation coefficient for the EAE of 9 % and of the HIG assay of 23 %. (VI)

Discussion

The immunochemical approach for measuring of C components offers a high degree of precision and specificity and analyses are fairly easily performed. The electroimmunoassay is more rapid than methods utilizing immunodiffusion. Complement factor D in clinical materials has previously been measured mainly using functional methods. One problem associated with functional assays concerns the possibility that other proteases might substitute for factor D (DiScipio, 1982) and cause falsely high factor D values. The low serum concentration of factor D, 1-2 mg/l, has so far been an obstacle to measuring D by ordinary immunochemical methods. The introduction of enzyme amplification

(Alpert et al., 1974) offered increased sensitivity of the electroimmunoassay comparable to that of radio-electroimmunoassay (Kindmark & Thorell, 1972) . Thus, the EAE permitted D protein to be measured at as low concentrations as 50 µg/l, several times lower than the normal serum concentration - with a precision clearly superior to that of the HIG assay for D. The results with EAE and of two functional assays for D agreed. Thus, no evidence of significant amounts of nonfunctional D protein was obtained in normal serum.

Davis et al. (1979) reported that factor D had γ-mobility in presence of Ca^{2+}or Mg^{2+}ions but β-mobility in the presence of EDTA, findings which agreed with observations of optimal anodal peaks with EDTA in the electrophoresis buffer. Although serial dilutions of NHS yielded linear dose-response curves in the assay, falsely low D values were obtained at extended dilution of serum in buffer where blurred immunoprecipitates appeared. The findings with purified D diluted in buffer were similar. Binding of factor D to other proteins (Konno et al., 1978), or possibly adsorption of factor D to the agarose, might explain the different appearance of precipitates obtained with dilution in D depleted serum and in buffer alone. It was recommended that serum samples with high D concentrations were diluted in suitable protein solutions, preferably D depleted serum.

The study suggest that among adults factor D concentrations vary little with age and sex; in children aged 2 - 10 years factor D concentrations are lower than in adults.

Fig 3.

Serum concentrations of complement factor D in 33 SLE patients at exacerbation (E) and at remission (R) of disease. I = 11 patients with mild disease, II = 11 patients with severe extra-renal disease and III = 11 patients with lupus glomerulonephritis. Values are given in per cent of normal. The reference area for factor D is 75 - 143 %.

SERUM LEVELS OF D PROTEIN IN SLE - RELATION TO DISEASE ACTIVITY AND RENAL FUNCTION (III, VII).

Methods, materials and results

The relationship between the serum concentrations of factor D protein, measured by EAE, and renal function was studied in 42 SLE patients (38 females and 4 males), 22 of whom had lupus glomerulonephritis. As a control group, 21 patients with a non-inflammatory renal disorder, polycystic kidney disease, were investigated (VII).

In patients with lupus glomerulonephritis the inverted values of D serum concentrations were closely correlated with the glomerular filtration rate (GFR) measured by Cr-EDTA clearance (Bröchner-Mortensen, 1972). Marked correlations were also found between the GFR and inverted values of two other low molecular weight (LMW) proteins, β_2-microglobulin and γ-trace. Among SLE patients without evidence of renal involvement, increased D serum concentrations were rare and the correlation between concentrations of factor D and S-creatinine did not reach significance in these patients. In the PCD group, close correlation was found between S-creatinine and each of the three LMW proteins ($r = 0.93 - 0.94$, $p<0.001$).

In 33 SLE patients serially studied during 10-30 months, factor D serum concentrations were largely unaffected by variations in disease activity (fig 3). Increased D values were found only in patients with lupus glomerulonephritis and factor D values were inversely correlated with GFR in these patients (III).

Discussion

The renal function has an important impact on the concentrations of LMW proteins in body fluids. Thus, these proteins are normally filtered freely in glomeruli and are absorbed and catabolized by the proximal tubules (Johansson & Ravnskov, 1972; Manuel et al., 1975). Consequently renal function might also be expected to exert an influence also on the serum concentrations of complement factor D particularly as D protein is present in the circulation as an active enzyme and is not consumed in the course of C activation (Lesavre & Müller-Eberhard, 1978).

By the studies of patients with lupus glomerulonephritis and polycystic kidney disease it was apparent that there is a close relationship between factor D concentrations in serum and renal function. Factor D concentrations were virtually uninfluenced of variation in disease activity in SLE and D protein concentration did not vary in relation to the inflammatory plasma protein response . Thus, the serum concentration of factor D are mainly determined by renal function.

A few patients reported in paper I had increased factor D hemolytic activity in serum despite their having apparently normal renal function. The explanation for this discrepancy is not evident, but may be related to the low precision of the HIG assay. Another explanation is the possible occurrence of enzymes such as kallikrein, in certain SLE sera and substituting for factor D in the HIG assay (Di Scipio, 1982). However, when a serum with pronounced discrepancy between HIG and EAE values was absorbed with anti-D immunosorbent the hemolytic factor D activity dissappeared (unpublished).

It has been proposed that serum concentrations of LMW proteins could be used as indices of renal impairment (Viberti et al., 1981). It is known that S-creatinine is an insensitive indicator

of GFR in rheumatic diseases (Nived et al., 1983). Since the serum concentrations of factor D was virtually unaffected by the extra-renal inflammatory process in SLE and was closely correlated with GFR, factor D could be a useful indicator of GFR in this disease.

GENERAL SUMMARY

The present investigations of the complement system in SLE were initiated with a pilot study of 8 SLE patients, six of whom had glomerulonephritis. The studies were extended to patients within a large SLE control program from which, on basis of active manifestations, patients were consecutively assigned to three clinical groups representing mild SLE, more severe extra-renal SLE and SLE glomerulonephritis. Thirty-three patients, followed for 10 - 30 months, in three equally large groups were investigated.

Hypocomplementemia with low values of C1q, C4 and C3 was usually associated with lupus glomerulonephritis, but was also sometimes seen in patients with extra renal disease. Persistently low concentrations of one or more classical pathway components at varying degrees of disease activity , possibly genetically determined , were found in 7 of 33 patients. One patient with a homozygous C2 deficiency was included in the study. Increased C1q concentrations during active disease were seen in 9 of 22 patients with extra-renal disease.

Of the alternative pathway components, P showed normal values and factor B normal or, particularly in extra-renal disease, increased concentrations. One observation of low B concentrations associated with the presence of Bb fragments in plasma was made in a patient who developed dissiminated intravascular coagulation during a severe flare with glomerulonephritis.

Hypocomplementemia was rare, among patients with pronounced inflammatory plasma protein response, as judged by CRP, α_1-antichymotrypsin, α_1-antitrypsin and orosomucoid serum

concentrations. Apparently the inflammatory process influenced the synthesis of C components such as C3 and C4. Particularly in lupus glomerulonephitis, CRP is sometimes insufficient as single variable of the acute phase reaction. Thus, CRP was undetectable in several patients, despite markedly high concentrations of other acute phase proteins.

C1, C2 and C3 activation was studied as estimated by serum concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ inactivator ($C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA) complexes, and the concentrations of C2a and C3d in EDTA-plasma. Evidence of C1 or C2 activation was noted in all patients during active disease, while C3d fragments were detected in increased amounts mainly in association with severe disease. Inverse correlations were found between immunochemical C2 concentrations and the percentage of cleaved C2, and between C3 and the C3 cleavage. The findings indicated that classical pathway activation was associated with accelerated C catabolism.

When serial samples were analyzed, increasing C1 activation was found to precede flares of extra-renal disease by several weeks. In patients with lupus glomerulonephritis, C3d could invariably be detected from the onset of exacerbations. Persistent presence of circulating C3d, from the onset of flares and throughout the observation period, was observed in three patients that developed renal failure. Clinical improvement was associated with decreasing concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA complexes and circulating C3d. C1q concentrations were transiently low during flares of lupus glomerulonephritis but otherwise the concentrations of C1q, C3 and C4 did not show consistent patterns of variation in relation to disease activity. Circulating immune complexes measured by solid phase assay, did not predict threatening flare but a decrease in serum concentrations of C1q binding immune complexes paralleled clinical improvement in patients with severe disease.

Complement factor D determined by hemolysis in gel was normal or high in 8 SLE patients with the highest concentrations being found in patients with reduced renal function. Using an enzyme

amplified staining technique, factor D in circulation could be quantified immunochemically by electroimmunoassay. Factor D values determined in 22 patients with lupus glomerulonephritis were correlated with S-creatinine. Glomerular filtration rate determined in 19 of the patients was inversely correlated with factor D concentrations. When factor D was measured in 33 sequentially followed SLE patients, high concentrations were exclusively found in those patients with renal involvement, and factor D did not vary in relation to disease activity and inflammatory plasma protein response. Thus, increased concentrations of factor D protein in SLE patients could be ascribed to retention due to reduced glomerular filtration.

ACKNOWLEDGEMENTS

The work upon which the present thesis is based has been carried out at the Department of Rheumatology and the Department of Immunology, Institute of Microbiology, University of Lund. The investigation was made possible through the kind help of many people. In particular I am indebted to:

Prof. Kåre Berglund, who introduced me to the field of rheumatological research. Dr Björn Svensson, for stimulating my interest in the immunology of rheumatic diseases and for inciting the SLE studies.

Prof. Anna-Brita Laurell and Prof. Frank Wollheim for their encouragement, constructive criticism and for providing a generous working athmosphere.

Dr Anders G.Sjöholm, my closest teacher, for expert guidance and stimulating discussion. Dr Ulf Johnson for invaluable help not least with computer programs.

Dr Lennart Truedsson and Dr Ola Nived, the closest of friends, for excellent collaboration and constant readiness to engage in constructive discussion.

Dr Hans Thysell for invaluable advice on nephrological problems. Bo Zöger for expert statistical advice.

Gunilla Nordin, Carina Linder, Ingrid Johansson, Birgitta Ellerström, Ulla Mårtensson, Eva Holmström and Kristina Nordström for their skilful technical assistance.

Ingrid Jönsson and Britt-Marie Karlsson for kind help with the typing of manuscripts. Elisabeth Möller for performing her

secreterial duties so cherfully.

Richard Pybus for instructive revision of the english text.

All the patients without whose cooperation this study had not been possible.

The investigations were supported by grants from the Swedish Medical Research Council (883-16X-68), the Medical Faculty, University of Lund, Konung Gustav V:s 80 års fond, Riksförbundet mot Reumatism, Greta och Johan Kochs Stiftelser, Thorsten och Elsa Segerfalcks Stiftelse, Alfred Österlunds Stiftelse, Förenade Liv, Ömsesidigt Gruppförsäkringsbolag, Stockholm, Kungliga Fysiografiska Sällskapets i Lund fonder.

Reproduction of the original papers was kindly permitted by S. Karger AG, Basel, Elsevier Science Publishers B.V., Amsterdam, Acta medica Scandinavica, Stockholm and Acta Pathologica, Microbiologica et Immunologica Scandinavica, Copenhagen.

REFERENCES

Aarden LA, deGrot ER & Feltkamp TEW: Crithidia luciliae, a simple substrate for the determination of anti-dsDNA with immunofluorescence technique. Ann. NY Acad. Sci. 254: 505, 1975.

Abrass CK, Nies KM, Louie JS et al.: Correlation and predictive accuracy of circulating immune complexes with disease activity in patients with systemic lupus erythematosus. Arth. Rheum. 23: 273, 1980.

Adu D & Cameron JS: Lupus nephritis. Clin. Rheum . Dis. 8: 153, 1982.

Agnello V: Complement deficiency states. Medicine 57:1, 1978.

Agnello V, Arbetter A, Ibanez de Kasep G et al.: Evidence for a subset of rheumatoid factors that cross-react with DNA-histon and have a distinct cross-idiotype. J. Exp. Med. 151:1514, 1980.

Aguado MT, Perrin LH, Ramirez R et al.: Evaluation of alternative pathway and factor B hemolytic activities in patients with systemic lupus erythematosus: correlations with the alternative pathway of regulatory proteins. Clin. Exp. Immunol. 42:495, 1980.

Alarcon-Segovia D: Cellular immunity and its regulation in SLE. Clin. Rheum. Dis. 8:63, 1982.

Albert DA, Hadler NM, & Ropes MW: Does corticosteroid therapy affect the survival of patients with systemic lupus erythematosus? Arth. Rheum. 22:945, 1979.

Alocer-Varela J & Alarcon-Segovia D: Decreased production of and response to interleukin-2 by cultured lymphocytes from patients with systemic lupus erythematosus. J. Clin. Invest. 69: 1388, 1982.

Alper CA, Colten HR, Rosen FS et al.: Homozygous deficiency of the third component of complement (C3) in a patient with repeated infections. Lancet ii:1179, 1972.

Alper CA et al.: Nomenclature of the alternative activating pathway of complement. Bull. WHO 59:489, 1981.

Alpert E, Coston R & Perrotto J: Immunoenzymatic assay for alpha-fetoprotein. Lancet, i:626, 1974.

Aronsen KF, Ekelund G, Kindmark C-O & Laurell CB: Sequential

changes of plasma proteins after surgical trauma. Scand. J. Clin. Lab. Invest. 29. Suppl 124:127, 1972.

Austen KF et al.: Nomenclature of complement. Immunochemistry 7:137, 1970.

Baehr G, Klemperer P & Schifrin A: A diffuse disease of the peripheral circulation usually associated with lupus erythematosus and endocarditis. Trans. Assoc. Am. Physicians 50:139, 1935.

Becker GJ, Waldburger M, Hughes GRV et al.: Value of serum C-reactive protein measurement in the investigation of fever in systemic lupus erythematosus. Ann. Rheum. Dis. 39:50, 1980.

Benedek TG & Rodnan GP: A brief history of rheumatic diseases. Bull. Rheum. Dis. 32:64, 1982.

Berglund K, Laurell A-B, Nived O, Sjöholm AG & Sturfelt G: Complement activation, circulating C1q binding substances and inflammatory activity in rheumatoid arthritis: relation and changes on suppression of inflammation. J. Clin. Lab. Immunol. 4:7, 1980.

Bertouch JV, Roberts-Thompson PJ, Feng PH et al.: C-reactive protein and serological indices of disease activity in systemic lupus erythematosus. Ann. Rheum. Dis. 42:655, 1983.

Bitter T: Systemic lupus erythematosus. Rheumatology 5:49, 1974.

Bokisch VA, Müller-Eberhard HJ & Cochrane CG: Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. J. Exp. Med. 129:1109, 1969.

Borsos T: Immunoglobulin classes and complement fixing activity. In Progress of Immunology (ed. Amos B) pp 841-848. Academic Press Inc., New York, 1971.

Brandslund I, Sierstad HC, Svehag S-E et al.: Double decker rocket immunoelectrophoresis for direct quantitation of complement C3 split products with C3d specificities in plasma. J. Immunol. Methods. 44:63, 1981.

Brandt L: Studies on the phagocytic activity of neutrophilic leucocytes. Scand. J. Haematol. Suppl 2, 1967. Thesis.

Brentjens JR & Andres GA: The pathogenesis of extrarenal lesions in systemic lupus erythematosus. Arth. Rheum. 25:880, 1982.

Bröchner-Mortensen J: A simple method for the determination of

glomerular filtration rate. Scand. J. Clin. Lab. Invest. 30:271, 1972.

Burnet FM: Autoimmune disease: II. Pathology of the immune response. Brit. Med. J. 2:720, 1959.

Canoso JJ & Cohen AS: A review of the use, evaluations, and criticisms of the preliminary criteria for the classification of systemic lupus erythematosus. Arth. Rheum. 22:917, 1979.

Chaplin H, Monroe MC & Lachmann PJ: Further studies of the C3g component of the α 2d fragment of human C3. Clin. Exp. Immunol. 51:639, 1982.

Charlesworth JA, Williams DG, Sherington E et al.: Metabolic studies of the third component of complement and the glycin-rich beta glycoprotein in patients with hypocomplementemia. J. Clin. Invest. 53:1578, 1974.

Christian CL: Systemic lupus erythematosus - clinical manifestations and prognosis. Arth. Rheum. 25:887, 1982.

Cluxton HE & Krause LAM: Acute lupus erythematosus disseminatus. Ann. Intern. Med. 19:843, 1943.

Cohen AS, Reynolds WE, Franklin CE et al.: Preliminary criteria for the classification of systemic lupus erythematosus. Bull. Rheum. Dis. 21:643, 1971.

Cooper NR: Enzymatic activity of the second component of complement . Biochemistry 14: 4245, 1975.

Cooper NR, Jensen FC, Welsh RM et al.: Lysis of RNA tumor viruses by human serum: Direct antibody-independent triggering of the classical pathway. J. Exp. Med. 144:970, 1976.

Cooper NR, Nemerow GR & Mayes JT: Methods to detect and quantitate complement activation. Springer Semin. Immunopathol. 6:195, 1983.

Coplon NS, Diskin CJ, Petersen J et al.: The long-term clinical course of systemic lupus erythematosus in end-stage renal disease. N. Engl. J. Med. 398(4): 186, 1983.

Daha MR, Hazevoet HM, Van Es LA et al.:Stabilization of the classical C3 convertase, $C\overline{42}$, by factor F-$\overline{42}$ isolated from serum of patients with systemic lupus erythematosus. Immunology 40:417, 1980.

Dameshek W: Systemic lupus erythematosus: A complex auto-immune disorder? Ann. Intern. Med. 48:707, 1958.

Davis P, Cumming RH & Verrier-Jones J: Relationship between anti-DNA antibodies, complement consumption and circulating immune complexes in systemic lupus erythematosus. Clin. Exp. Immunol. 28:226, 1977.

Davis III AE, Zalut C, Rosen FS et al.: Human factor D of the alternative complement pathway. Physicochemical characteristics and N-terminal amino sequence. Biochemistry 18:5082, 1979.

DiScipio RG: The activation of the alternative pathway C3 convertase by human plasma kallikrein. Immunology 45:578, 1982.

Dixon F: Murine lupus - an overview. Arth. Rheum. 25:721, 1982.

Donaldsson VH & Evans RR: A biochemical abnormality in hereditary angioneurotic edema: absence of serum inhibitor of C1 esterase. Amer. J. Med. 35:37, 1963.

Donaldsson VH, Hess EV & McAdam AJ: Lupus erythematosus-like disease in three unrelated women with hereditary angioneurotic edema. Ann. Intern. Med. 86:312, 1977.

Dubois EL, Wierzchowiecki M, Cox MB et al.: Duration and death in SLE: an analysis of 249 cases. J. Am. Med. Assoc. 227:1399, 1974.

Estes D & Christian CL: The natural history of systemic lupus erythematosus by prospective analysis. Medicine 50:85, 1971.

Fearon DT, Austen KF & Ruddy S: Formation of a hemolytically active cellular intermediate by the interaction between properdin factors B and D and the activated third component of complement. J. Exp. Med. 138:1305, 1973.

Fearon DT & Austen KF: Properdin: binding to C3b and stabilization of the C3b dependent C3 convertase. J. Exp. Med. 142:856, 1975.

Fearon DT: Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. Proc. Natl. Acad. Sci. 76:5867, 1979.

Fearon DT: Complement. J. Allergy Clin. Immunol. 71:520, 1983.

Fearon DT & Wong WW: Complement ligand-receptor interactions that mediate biological responses. Ann. Rev. Immunol. 1:243, 1983.

Fessel WJ: Systemic lupus erythematosus in the community. Incidence, prevalence, outcome, and first symptoms, the high prevalence in black women. Arch. Intern. Med. 134:1027, 1974.

Fey G & Colten HR: Biosynthesis of complement components. Fed. Proc. 40:2099, 1981.

Fiedler AHL, Walport MJ, Batchelor JR et al.: Family study of the major histocompatibility complex in patients with systemic lupus erythematosus: importance of null alleles of C4A and C4B in determining disease susceptibility. Brit. Med. J. 286:425, 1983.

Forbes CD, Pensky J & Ratnoff OD: Inhibition of activated Hageman factor and activated plasma tromboplastin antecedant by purified serum C1 inactivator. J. Lab. Clin. Med. 76:809, 1970.

Frank MM, Gelfand JA & Atkinson JP: Hereditary angioedema: the clinical syndrome and its management. Ann. Intern. Med. 84:580, 1976.

Ganrot PO: Crossed immunoelectrophoresis. Scand. J. Clin. Lab. Invest. 29, suppl 124:39, 1972.

George D & Glass D: Quantitation of complement proteins in rheumatic diseases. Clin. Rheum. Dis. 9:177, 1983.

Gigli I & Austen KF: Fluid phase destruction of $C2^{hu}$ by $C1^{hu}$. I. Its enhancement and inhibition by homologous and heterologous C4. J. Exp. Med. 129:679, 1969.

Glass DN, Raum D, Gibson D et al.: Inherited deficiency of the second component of complement. Rheumatic disease associations. J. Clin. Invest. 58:853, 1976.

Glass DN, Fearon DT & Austen KF: Inherited abnormalities of the complement system. In:The Metabolic Basis of Inherited Disease. Eds. Stanburg JB, Wyngaarden JB, Frederickson DS et al. p. 1934. Mc Graw-Hill, New York, 1983.

Grigor R, Edmonds J, Lewkonia R et al.: Systemic lupus erythematosus. A prospective analysis. Ann. Rheum. Dis. 37:121, 1978.

Hadler M, Gerwin RD, Frank MM et al.: The fourth component of complement in the cerebrospinal fluid in systemic lupus erythematosus. Arth. Rheum. 16:507, 1973.

Hahn BH: Cell-mediated immunity in systemic lupus erythematosus. Clin. Rheum. Dis. 1:497, 1975.

Hannestad K & Stollar BD: Certain rheumatoid factors react with nucleosomes. Nature 275:671, 1978.

Hargraves MM: Production *in vitro* of LE cell phenomenon: Use of normal bone marrow elements and blood plasma from patients

with acute disseminated lupus erythematosus. Proc. Staff. Meet. Mayo Clin. 24:234, 1949.

Harpel PC & Cooper NR: Studies on human plasma $C\bar{1}$ inactivator-enzyme interactions. J. Clin. Invest. 55:593, 1975.

Harris EN, Gharavi AE, Boey ML et al.: Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. Lancet, ii:1211, 1983.

Harvey AM, Schulman LE, Tumulty A et al.: Systemic lupus erythematosus: Review of the litterature and clinical analysis of 138 cases. Medicine (Baltimore)33:291, 1954.

Hauck L: The positive reaction of the Wassermann-Neisser-Bruck test in acute lupus erythematosus. München Med. Wschr. 57:17, 1910.

Hedlund P: The appearance of acute phase protein in various diseases. Acta Med. Scand. 128:579, 1947.

Honig S, Gorevic P & Weissman G: C-reactive protein in systemic lupus erythmatosus. Arth. Rheum. 20:1065,1977.

Hughes GRV: The treatment of SLE: the case for conservative management. Clin. Rheum. Dis. 8:299, 1982.

Hunder GG, Mullen BJ, & McDuffie FC: Complement in pericardial fluid of systemic lupus erythematosus. Ann. Intern. Med. 80:453, 1974.

Iida K & Nussenzweig V: Complement receptor is an inhibitor of the complement cascade. J. Exp. Med. 153:1138, 1981.

Iida K, Mornaghi R & Nussenzweig V: Complement receptor (CR1) deficiency in erythrocytes from patients with systemic lupus erythematosus. J. Exp. Med. 155:1427, 1982.

Inman RD: Immune complexes in SLE. Clin. Rheum Dis. 8:49, 1982.

Jarret MP, Sablay LB, Walter L et al.: The effect of continous normalization of serum hemolytic complement on the course of lupus nephritis. A five year prospective study. Am. J. Med. 70:1067, 1981.

Johansson BG & Ravnskov U: The serum level and urinary excretion of α_2-microglobulin, β_2-microglobulin and lysozyme in renal disase. Scand. J. Urol. Nephrol. 6: 249, 1972.

Johnson U, Ohlson K & Olsson I: Effects of granulocyte neutral proteases on complement components. Scand. J. Immunol. 5:421,

1976.

Johnson U & Holmström E: C3 fragmentation in human serum : formation of a mixed disulphide between C3d and albumin. Acta path. microbiol. immunol. scand Sect C. 90: 321, 1982.

Johnson U: Influence of ageing and polyethylene glycol on the quantitation of C3d/dg in plasma and serum. Acta path. microbiol. immunol. scand. Sect C. In press.

Kaplan D: Systemic lupus erythematosus - corticosteroids. Clin Rheum. Dis. 9:601, 1983.

Karsh J, Klippel JH, Balow JE et al.: Mortality in lupus nephritis. 22: 764, 1979.

Kazatchkine MD, Fearon DT & Austen KF: Human alternative complement pathway: membrane associated sialic acid regulates the competition between B and beta-1-H for cell bound C3b. J. Immunol. 122:75, 1979.

Kazatchkine MD & Nydegger UE: The human alternative complement pathway: Biology and immunopathology of activation and regulation. Prog. Allergy. 30:193, 1982.

Keil H: Conception of lupus erythematosus and morphologic variants with particular reference of "systemic" lupus erythematosus. Arch. Derm. (Chicago) 36:729, 1937.

Kerr MA: The human complement system: assembly of the classical pathway C3 convertase. Biochem. J. 189:173, 1980.

Kindmark C-O & Thorell JI: Quantitative determination of individual serum proteins by radio-electroimmunoassay and use of 125I-labelled antibodies (application to C-reactive protein) Scand. J. Clin. lab. Invest. 29, Suppl 124 : 49, 1972.

Klemperer P, Pollack AD & Baehr G: Pathology of disseminated lupus erythematosus. Arch. Path. (Chicago) 32:569, 1941.

Knobel HR, Heusser MLR & Isliker H: Enzymatic digestion of the first component of complement (C1q). J. Immunol. 112:2094, 1974.

Konno T, Katsuno Y & Hirai H: Change of electrophoretic mobility of purified alternative pathway factor D. J. Immunol. Methods 21:325, 1978.

Kushner I: The phenomenon of the acute phase response. In C-reactive protein and the plasma protein response to tissue injury. Eds. Kushner I, Volanakis JE & Gewurz H. p. 39. Annals of the New York Academy of Sciences. Vol 389, 1982.

Lachmann PJ & Hobart MJ: Complement technology. In: Weir DM (Ed) Handbook of experimental immunology, vol. 1. Immunochemistry. Blackwell Scientific Publications. Oxford 1978. Chapt 5A.

Lachmann PJ & Peters DK: Complement. In Clinical aspects of immunology. Ed. by Lachmann PJ, Peters DK. Blackwell Scientific Publications, Oxford. p 18, 1982.

Laurell A-B, Mårtensson U & Sjöholm AG: C1 subcomponent complexes in normal and pathological sera studied by crossed immunoelectrophoresis. Acta path microbiol scand. Sect C 84:455, 1976.

Laurell A-B, Johnson U, Mårtensson U & Sjöholm AG: Formation of complexes composed of $C\bar{1}r$, $C\bar{1}s$ and $C\bar{1}$-inactivator in human serum on activation of C1. Acta path.microbiol. immunol. scand. Sect C. 86:299, 1978a.

Laurell A-B, Mårtensson U & Sjöholm A: The development of simple tests for C1q, C1r, C1s,C2 and properdin. In Clinical aspects on the complement system. Eds Opferkuch W, Rother K, Schultz DR. Int. Symp. Bochum 1976 p 12, Georg Thieme Publishers, Stuttgart, 1978b.

Laurell A-B, Mårtensson U & Sjöholm AG: Quantitation of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ inactivator complexes by electroimmunoassay. Acta path microbiol. scand. Sect C,87:79, 1979.

Laurell CB: Electroimmunoassay. Scand. J. Clin. Lab. Invest. suppl. 124:21, 1972.

Lee P, Urowitz MB, Bookman AAM et al.: Systemic lupus erythematosus. A review of 110 cases with reference to nephritis, the nervous system, infections, aseptic necrosis and prognosis. Quarterly J. Medicine 46:1, 1977.

Leonhardt T: Family studies in systemic lupus erythematosus. Acta Med. Scand. 176, suppl 416, 1964.

Lepow IH, Naff GB, Todd EW et al.: Chromatographic resolution of the first component of complement into three activities. J. Exp. Med. 117:983, 1963.

Lesavre PH & Müller-Eberhard HJ: Mechanism of action of factor D of the alternative complement pathway. J. Exp. Med. 148:1498, 1978.

Lesavre PH, Hugli TH, Esser AF et al.: The alternative pathway C3/C5 convertase: chemical basis of factor B activation. J. Immunol. 123:529, 1979.

Libman E & Sacks B: A hitherto undescribed form of valvular and

mural endocarditis. Arch. Intern. Med. 33:701, 1924.

Lightfoot RW & Hughes GRV: Significance of persisting serologic abnormalities in SLE. Arth. Rheum. 19:837, 1976.

Lloyd W & Schur P: Immune complexes, complement and anti-DNA in exacerbations of systemic lupus erythematosus (SLE). Medicine 60:208, 1981.

Loos M: The classical complement pathway: mechanism of activation of the first component by antigen-antibody complexes. Prog Allergy 30:135, 1982.

Löfberg H & Grubb AO: Quantitation of γ-trace in human biological fluids; indications for production in the central nervous system. J. Clin. Lab. Invest. 39:619, 1979.

Mannik M: Pathophysiology of circulating immune complexes. Arth. Rheum. 25:783, 1982.

Manuel Y, Colle A & Leclercq M et al.: Low molecular weight proteinuria. Contrib. Nephrol. 1:156,1975.

Martin A, Lachmann PJ, Halbwachs L et al.: Hemolytic diffusion plate assays for factors B and D of the alternative pathway of complement activation. Immunochemistry 13:317, 1976.

Mayes JT, Schreiber RD & Cooper NR: Development and application of an enzyme linked immunosorbent assay for the quantitation of alternative complement pathway activation in human serum. Clin. Res. 31:539, 1983.

Meddings J & Grennan DM: The prevalence of systemic lupus erythematosus (SLE) in Dunedin. 91:205, 1980.

Miller ML & Steinberg AD: Systemic lupus erythematosus - immunoregulatory therapies. Clin. Rheum. Dis. 9: 617, 1983.

Morimoto C, Reinherz EL, Schlossman SF et al.: Alterations in immunoregulatory T cell subsets in active systemic lupus erythematosus. J. Clin. Invest. 66:1171, 1980.

Morrow WJW, Williams DJP, Ferec C et al: The use of C3d as a means of monitoring clinical activity in systemic lupus erythematosus and rheumatoid arthritis. Ann. Rheum. Dis. 42:668, 1983.

Müller-Eberhard HJ, Polley MJ & Calcott MA: Formation and functional significance of a molecular complex derived from the second and fourth component of human complement. J. Exp. Med. 125:359, 1967.

Müller-Eberhard HJ & Götze O: C3 proactivator convertase and its mode of action. J. Exp. Med. 135;1003, 1972.

Müller-Eberhard HJ & Schreiber RD: Molecular biology and chemistry of the alternative pathway of complement. Adv. Immunol. 29:1, 1980.

Nagasawa S & Stroud RM: Cleavage of C2 by $C\bar{1}s$ into antigenically distinct fragments C2a and C2b: Demonstration of binding of C2b to C4b. Proc. Natl. Acad. Sci. USA. 74:2998, 1977.

Ngan BY, Gelfand EW & Minta JO: A simple one-step hemolytic assay for C2 with C2-deficient human serum. J. Immunol. 118:736, 1977.

Nicholson A, Brade V, Schorlemmer H-U et al.: Interaction of C3b, B and D in the alternative pathway of complement activation. J. Immunol. 115:1108, 1975.

Nived O, Sturfelt G, Westling H & White T: Is serum creatinine a reliable index of renal function rheumatic diseases? Brit. Med. J. 286:684, 1983.

Nived O, Sturfelt G & Wollheim F: Systemic lupus erythematosus in adult population of southern Sweden: incidence, prevalence and validity of classification criteria. To be published.

Ochs HD, Wedgwood RJ, Frank MM et al.: The role of complement in the induction of antibody responses. Clin. Exp. Immunol. 53: 208, 1983.

Osler W: On the visceral manifestations of the erythema group of skin diseases. Trans. Ass. Amer. Phychs. 18:599, 1903.

Pangburn MK, Schreiber RD & Müller-Eberhard HJ: Human complement C3b inactivator: Isolation, characterization, and demonstration of an absolute requirement for the serum protein β1H for cleavage of C3b and C4b in solution. J. Exp. Med. 146:257, 1977.

Pangburn MK & Müller-Eberhard HJ: Relation of a putative thioester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. J. Exp. Med. 152:1102, 1980.

Pepys MB: C-reactive protein : the role of an ancient protein in modern rheumatology. Clin. Exp. Rheumatology. 1:3, 1983.

Perrin LH, Lambert PH & Miescher PA: Complement breakdown products in plasma from patients with systemic lupus erythematosus and patients with membranoproliferative or other glomerulonephritis. J. Clin. Invest. 56:165, 1975.

Petersen BH, Lee TJ, Snyderman R et al.: Neisseria meningitidis and Neisseria gonorrhoeae bacteremia associated with C6, C7 or C8 deficiency. Ann. Intern. Med. 90:917, 1979.

Petz LD, Powers R, Fries JR et al.: The in vivo metabolism of the third component of complement in systemic lupus erythematosus. 20:1304, 1977.

Pincus T: Studies regarding a possible function for viruses in the pathogenesis of systemic lupus erythematosus. Arth. Rheum. 25:847, 1982.

Podack ER, Kolb WP, Müller-Eberhard HJ: The C5b-6 complex: formation, isolation and inhibition of its activity by lipoprotein and the S-protein of human serum. J. Immunol. 120:1841, 1978.

Podack ER, Esser AF, Biesecker G et al.: Membrane attack complex of complement: A structural analysis of its assembly. J. Exp. Med. 301, 1980.

Podack ER, Preissner KT & Müller-Eberhard HJ: Inhibition of C9 polymerization within the SC5b-9 complex of complement by S-protein. Acta path. microbiol. immunol. scand. Sect. C Suppl. 92: 89, 1984.

Polley MJ & Müller-Eberhard HJ: The second component of human complement: its isolation, fragmentation by C'1 esterase, and incorporation into C'3 convertase. J. Exp. Med. 128:533, 1968.

Porter RR & Reid KBM: Activation of the complement system by antibody-antigen complexes: the classical pathway. Adv. Prot. Chem. 33:1, 1979.

Ratnoff OD & Naff GB: The conversion of C'1s to C'1-esterase by plasmin and trypsin. J. Exp. Med. 125:337, 1967.

Ratnoff OD, Pensky J, Ogston D et al.: The inhibition of plasmin, plasma kallikrein, plasma permeability factor, and the C'1r subcomponent of the first component of complement by serum C'1 esterase inhibitor. J. Exp. Med. 129:315, 1969.

Reda MG & Baigelman W: Pleural effusion in systemic lupus erythematosus. Acta Cytologica 24:553, 1980.

Reid KBM & Porter RR: Subunit composition and structure of sub-component C1q of the first component of human complement. Biochem. J. 155:19, 1976.

Rosner S, Ginzler EM, Diamond HS et al.: A multicenter study of outcome in systemic lupus erythematosus. II. Causes of Death. Arth. Rheum. 25:612, 1982.

Ross GD: Structure and function of membrane complement receptors. Fed. Proc. 41:3089, 1982.

Ruddy S & Austen KF: natural control mechanisms of the complement system. In Proceedings of the International Symposium on the Biological Activities of Complement. Ed. Ingram R. p 13. S.Karger, Basel 1972.

Rynes RI: Inherited complement deficiency states and SLE. Clin. Rheum. Dis 8:29, 1982.

Scheidegger JJ: Une micro-methode l'immunoelectrophorese. Int. Arch. Allergy. 7:103, 1955.

Schur : Complement in lupus. Clin. Rheum. Dis. 1: 519, 1975.

Schur P: Complement and lupus erythematosus. Arth. Rheum. 25:793, 1982.

Schutte M, DiCamelli R, Murphy P et al.: Effects of anestesia, surgery and inflammation upon host defence mechanisms. I. Effects upon the complement system. Int. Archs. Allergy appl. Immun. 48:706, 1975.

Scullion M, Balint G & Whaley K: Evaluation of the C1q solid-phase binding assay for immune complexes. A clinical and laboratory study. J. Clin. Lab. Immunol. 2:15, 1979.

Shelton E, Yonemasu K & Stroud RM: Ultrastructure of the human complement, C1q. Proc. Natl. Acad. Sci (Wash) 69:65, 1972.

Sibbit WL, Likar L, Spellman CW et al.: Impaired natural killer cell function in systemic lupus erythematosus. Relationship to interleukin-2 production. Arth. Rheum. 26: 1316, 1983.

Siegel J, Rent R & Gewurz H: Interactions of C-reactive protein with the complement system. I. Protamine-induced consumption of complement in acute phase sera. J. Exp. Med. 140:631, 1974.

Siegel J, Osmand AP, Wilson MF et al.: Interactions of C-reactive protein with the complement system. II. C-reactive protein mediated consumption of complement by poly-L-lysine polymers and other polycations. J. Exp. Med. 142, 709, 1975.

Sjöholm AG: Complement components in normal serum and plasma quantitated by electroimmunoassay. Scand. J. Immunol. 4:25, 1975.

Sturfelt G, Linder C, Sjöholm AG & Svensson B: Impaired monocyte motility in sera from patients with systemic lupus erythematosus. Acta path. microbiol. immunol. scand Sect C 91:

151, 1983.

Svensson B: Monocyte in vitro function in systemic lupus erythematosus (SLE). I. A clinical and immunological study. Scand. J. Rheumatol. Suppl. 31:29, 1980.

Swaak AJG, Aarden LA, Statius van Eps LW et al.: Anti-dsDNA and complement profiles as prognostic guides in systemic lupus erythematosus. Arth. Rheum. 22:226, 1979.

Takahashi M, Takahashi S & Hirose S: Solubilization of antigen-antibody complexes: a new function of complement as a regulator of immune reactions. Prog. Allergy 27:134, 1980.

Talal N: Sex hormones and modulation of immune response in SLE. Clin. Rheum. Dis. 8:23, 1982.

Talbott JH: Historical background of discoid and systemic lupus erythematosus. In: Lupus erythematosus. Ed. Dubois EL, pp 1-9. University of Southern California Press. Los Angeles California (1974).

Tan EM & Kunkel HG: Characteristics of soluble antigen precipitating with sera of patients with systemic lupus erythematosus. J. Immunol. 96: 464, 1966.

Tan EM, Cohen AS, Fries JF et al.: The 1982 revised criteria for the classification of the systemic lupus erythematosus. Arth. Rheum. 25:1271, 1982.

Taylor JC, Crawford IP & Hugli TE: Limited degradation of the third component (C3) of human complement by human leukocyte elastase (HLE): partial characterization of C3 fragments. Biochemistry 16:3390, 1977.

Teisner B, Brandslund I, Hau J et al.: Heterogeneity in electrophoretic mobility of C3-derived molecules expressing D but not C-epitopes following *in vivo* activation of the complement system. Acta path. microbiol. immunol. scand. Sect C 91: 85, 1983.

Tron F & Bach JF: Relationships between antibodies to native DNA and glomerulonephritis in systemic lupus erythematosus. Clin. Exp. Immunol. 28:426, 1977.

Truedsson L, Sjöholm AG & Laurell A-B: Screening for deficiencies in the classical and alternative pathways of complement by hemolysis in gel. Acta path. microbiol. scand. Sect. C 89:161, 1981.

Ueda A, Kusaba T & Yanase T: Determination of the second component of complement (C2) by electroimmunoassay in sera from

patients with systemic lupus erythematosus. Clin. Exp. Immunol. 53:217, 1983.

Urowitz MB, Bookman AAM, Koehler BE et al.: The bimodal mortality pattern of systemic lupus erythematosus. Am. J. Med. 60:221, 1976.

Vaughan JH, Bayles TB & Savour CB: The response of serum gammaglobulin level and complement titer to adrenocorticotrophic hormone therapy in lupus erythematosus disseminatus. J. Lab. Clin. Med. 37:698, 1951.

Viberti GC, Keen H & Mackintosh D: Beta$_2$ -microglobulinemia: a sensitive index of diminishing renal function in diabetics. Brit. Med. J. 282:95, 1981.

Vogt W, Dames W, Schmidt G et al.: Complement activation by the properdin system: formation of a stoichiometric C3 cleaving complex of properdin factor B with C3b. Immunochemistry 14:201, 1977.

Vogt NG, Schmidt B, Buttlar HB van et al.: A new function of the activated third component of complement: binding to C5, an essential step for C5 activation. Immunology 34:29, 1978.

Volonakis JE, Schrohenloher RE & Stroud RM: Human factor D of the alternative complement pathway: purification and characterization. J. Immunol. 119:337, 1977.

Volonakis JE: Complement activation by C-reactive protein complexes. In: C-reactive protein and the plasma protein response to tissue injury. Ed. Kushner I, Volonakis JE and Gewurz H. p 235. The New York Academy of Sciences. 1982.

Walport MJ, Black CM & Batchelor JR: The immunogenetics of SLE. Clin. Rheum. Dis. 8:3, 1982.

Westberg NG, Naff GB, Boyer JT et al.: Glomerular deposition of properdin in acute and chronic glomerulonephritis with hypocomplementemia. J. Clin. Invest. 50:642, 1971.

Whaley K, Schur P & Ruddy S: Relative importance of C3b inactivator and β1H globulin in the modulation of the properdin amplification loop in systemic lupus erythematosus. Clin. Exp. Immunol. 36: 408, 1979.

Whaley K, Lappin D & Hamilton AO: Serum treated antigen-antibody complexes inhibit the production of C2 and factor B by mononuclear phagocytes. Immunology 48:255, 1983.

Williams DG, Peters DK, Fallows J et al.: Studies of serum complement in the hypocomplementemic nephritides. Clin. Exp.

Immunol. 18:391, 1974.

Wilson MR, Arroyave CM, Nakamura R et al.: Activation of the alternative pathway of complement in systemic lupus erythematosus. Clin. Exp. Immunol. 26:11, 1976.

Ziccardi RJ & Cooper NR: Activation of C1r by proteolytic cleavage. J. Immunol. 116:504, 1976.

Ziccardi RJ & Cooper NR: The subunit composition and sedimentation properties of human C1. J. Immunol. 118:2047, 1977.

Ziccardi RJ: The first component of human complement. Springer Semin. Immunopathol. 6:213, 1983.

Zubler RH, Lange G, Lambert PH et al.: Detection of immune complexes in unheated sera by a modified 125-I-C1q binding test. Effect of heating on the binding of C1q by immune complexes and application of the test to systemic lupus erythematosus. J. Immunol. 116:232, 1976.

APPENDIX (I-VII)

International Archives of Allergy and Applied Immunology

Editors-in-Chief: R.R.A. Coombs, Cambridge;
L.Å. Hanson, Göteborg; P. Kallós, Helsingborg;
F. Milgrom, Buffalo, N.Y.; Z. Trnka, Basel; G.B. West, Epsom

Publishers: S. Karger, Basel
Reprint (Printed in Switzerland)

Int. Archs Allergy appl. Immun. *70:* 12–18 (1983)

© 1983 S. Karger AG, Basel
0020–5915/83/0701–0012 $ 2.75/0

Complement Components, C1 Activation and Disease Activity in SLE

G. Sturfelt, A.G. Sjöholm, B. Svensson

Department of Rheumatology and Institute of Medical Microbiology, Department of Immunology, University of Lund, Sweden

Abstract. Laboratory parameters were studied in 8 systemic lupus erythematosus patients during periods of high and low disease activity, mainly as defined by clinical criteria. Renal manifestations were present in 6 patients 5 of which showed antibodies to native DNA. C-reactive protein was raised in 3 patients. Only 1 of these showed a superimposed bacterial infection. Markedly high concentrations of C1r-C1s-C1 inactivator complexes (C1r-C1s-C1 IA) in the sera provided direct evidence of C1 activation independent of disease activity. During active disease, C1r-C1s-C1 IA were correlated with C1q binding immune complexes as measured by solid phase, but not by fluid phase assay. Immunochemical concentrations of C1q, C4 and C3 and functional C2 were decreased in active SLE, consistent with sequential activation of the classical pathway. Discrepancies were noted between functional and immunochemical assays for C2 but not for factor B. Although essentially within the normal range, the levels of C1s, C4 binding protein, C5 and properdin were lower during active than during inactive disease. The concentrations of the factors B, I and H did not suggest involvement of the alternative pathway. 1 exceptional patient showed low factor B, a relative decrease of factor I and the presence of Bb fragments in plasma during active SLE. Markedly high factor D values were found. This could partly be explained by reduced renal function.

Introduction

Complement (C) activation by immune complexes is considered an important pathogenetic mechanism in systemic lupus erythematosus (SLE), providing a rationale for the use of C and immune complex assays in monitoring of the disease [8]. It is well known that serum levels of classical pathway components are low in active SLE [7, 22]. Evidence has also been given for involvement of the alternative pathway and of regulator proteins of the C system [3, 19–21, 29, 31–33].

In the present investigation laboratory parameters were compared during active disease and during remission in 8 SLE patients. The formation of complexes between C1r, C1s and C1 IA was measured as a means for direct assessment of C1 activation. Other parameters examined included C1q binding immune complexes, C component levels, factor B fragments and the concentrations of C-reactive protein (CRP).

Classical pathway components were designated numerically, while alternative pathway components were given by capital letters as recommended by nomenclature committees sponsored by the World Health Organization [Bulletin of the World Health Organization *39:* 935 (1968); Bulletin of the World Health Organization *59:* 489 (1981)]. Fragments of C components are indicated by small letters following the component symbol. Other abbreviations are given in the text.

Materials and Methods

Patients

8 patients were included in the study. The patients satisfied 5–9 criteria proposed by the American Rheumatism Association (ARA) for the diagnosis of SLE [6]. An immunofluorescent test for antinuclear antibodies replaced the LE-cell test as a diagnostic criterium.

The patients were followed at the Department of Rheumatology, University Hospital, Lund, for 2–5 years (mean 3 years). Clinical examinations and blood sampling were carried out at intervals of 2–8 months. Serum and EDTA-plasma samples were stored in aliquots at –80 °C.

Table I. Clinical data in the 8 SLE-patients during high (H) and low (L) disease activity

Patient	Sex	Age at onset	Age at 1st examination		Clinical features	Medication per day	
I	♀	21	23	H	fever, pleuritis, arthritis	–	
				L	good general condition; no sign of active organ involvement	prednisolone	17.5 mg
II	♂	30	33	H	arthritis, rash	–	
				L	no organ involvement	–	
III	♂	45	45	H	fever, glomerulonephritis, arthritis, pericarditis, bronchopneumonia	–	
				L	good general condition; no sign of active organ involvement	betamethazone aspirin	3 mg 3 g
IV	♀	22	22	H	fever, bad general condition, glomerulonephritis, pericarditis, arthritis, disseminated intravascular coagulation	hydrocortison	1 g
				L	good general condition; renal function stable	deltison	25 mg
V	♀	26	28	H	fever, rash, stomatitis, glomerulonephritis, psychosis	prednisolone	7.5 mg
				L	good general condition, low active rash; renal function stable	betamethazone	1.0 mg
VI	♂	25	26	H	moderately affected general condition, rash, pleuritis, glomerulonephritis, arthritis	–	
				L	good general condition; renal function stable	prednisolone	32.5 mg
VII	♀	15	30	H	moderately affected general condition, rash, arthritis, glomerulonephritis; proteinuria > 1 g/day	azathioprine prednisolone	50 mg 10 mg
				L	good general condition; renal function stable; proteinuria < 1 g/day	betamethazone cyclophosphamide	1.5 mg 50 mg
VIII	♀	25	29	H	rash, arthritis, glomerulonephritis, general condition moderately affected	prednisolone	10 mg
				L	good general condition; renal function stable	prednisolone azathioprine	10 mg 100 mg

Based on the clinical data as summarized in table I one episode of high and one of low disease activity were selected for each patient. The evaluation was made independently by two of the authors. Assessment of renal function included repeated measurement of creatinine in serum and of the glomerular filtration rate (GFR; table II).

Renal involvement with glomerulonephritis as suggested by urine analysis was present in 6 patients (III–VIII). Neuropsychiatric manifestations were present in 1 patient (V). 2 patients (I and II) showed more benign SLE syndromes.

Immunochemical Complement Assays

C1q, C1s, C2, C4, C3, C5, P, B and C1 inactivator (C1 IA) were determined as described earlier [13, 25]. The electroimmunoassay [16] was also used for measurement of the factors I and H and of C4 binding protein (C4bp). EDTA at 2 mmol/l was included in the electrophoresis buffers. The assay for C4bp required rapid application of samples after dilution. Values were given as percentages of the concentration in a reference serum or an EDTA-plasma pool said to contain each component at 100%. The reference areas (95% confidence intervals) were based on determinations in 100 healthy

Table II. Hematological parameters, acute phase reactants, IgG levels, antibody titers to native DNA (anti-nDNA) and tests for renal function, compared at high (H) and at low (L) disease activity in 8 SLE patients

Patient		Hemoglobin	Lymphoc. perif. blood	ESR	C-reactive protein	IgG	anti-nDNA	creatinine	GFR
I	H	61	0.17	128	188	27.6	80	40	not done
	L	142	1.60	5	12	22.6	40	55	not done
II	H	137	2.61	36	12	28.5	0	80	not done
	L	134	0.94	33	12	20.0	0	80	not done
III	H	106	1.43	73	32	17.1	40	80	80
	L	113	1.44	29	12	16.5	20	90	80
IV	H	126	0.91	9	13	17.7	10	170	51
	L	127	3.91	30	12	10.4	0	85	56
V	H	101	0.38	44	12	24.5	320	75	114
	L	127	0.99	35	12	18.9	160	60	100
VI	H	119	0.96	75	105	19.0	640	105	111
	L	148	1.15	15	12	11.4	0	80	124
VII	H	111	0.42	81	12	6.1	0	80	54
	L	96	0.32	90	12	4.9	0	100	75
VIII	H	109	0.50	129	12	17.0	320	120	76
	L	99	0.57	68	12	10.0	0	210	24
Reference area		131–163 g/l	1.2–4.0 10^9/l	2–15 mm	< 12 mg/l	7.0–15.0 g/l	< 10 titer	60–115 μmol/ml	> 80 ml plasma/min/1.73 m^2
Difference between values at H and at L disease activity		n.s.	n.s.	n.s.	n.s.	$p < 0.01$	n.s.	–	–

n.s. = Not significant.

adults. For C4bp the observed range in 25 healthy adults was used.

Complexes of C1r, C1s and C1 IA (C1r-C1s-C1 IA) were estimated by electroimmunoassay and values were given in percent of C1r-C1s-C1 IA in a normal serum pool after activation of C1 with heat-aggregated IgG [15]. In normal sera the levels of C1r-C1s-C1 IA ranged between 11 and 25% of the reference.

Bb fragments of B were measured by electroimmunoassay using high electroendosmotic agarose (H.E. Marine Colloids, Research Products, Miles Laboratories, Stoke Pages, England). EDTA-plasma was applied undiluted or diluted 1:5. The electrophoresis was carried out for 16 h at 15 v/cm in 75 m*M* barbital buffer, pH 8.6, containing EDTA at 2 mmol/l. Cathodal precipitates were developed with an antiserum produced against Bb fragments. Values were given as a percentage of Bb in a serum pool incubated with inulin at 100 mg/ml for 5 h, after which the serum pool was said to contain Bb at 100%.

Functional Complement Assays

Screen tests for the classical and the alternative pathways were performed as reported previously [28]. B and D were determined essentially as described by *Martin* et al. [17]. Functional C2 was determined by hemolysis in gel using C2-deficient serum according to *Lachmann and Hobart* [11].

Circulating Immune Complexes

Circulating immune complexes were measured by a fluid phase C1q binding assay (C1qBA) according to *Zubler* et al. [30] and by a solid phase C1q binding assay (C1qSP) mainly as described by *Scullion* et al. [23]. Following the initial incubation at 37°C for 1 h, samples

were incubated at 4 °C for 20 h with ^{125}I-C1q and solid phase C1q, respectively. In the C1qSP sheep anti-IgG (Statens Bakteriologiska Laboratorium, SBL, Stockholm, Sweden) was used after labelling with ^{125}I. In both assays values were expressed as equivalents of aggregated IgG in micrograms per milliliter. Control sera yielded values < 100 μg/ml in the C1qBA and < 10 μg/ml in the C1qSP.

Other Assays

Anti-DNA antibodies were determined by the *Crithidia Luciliae* test [1]. IgG and CRP were determined by electroimmunoassay [16]. For CRP the electrophoresis was carried out in the presence of EDTA. Erythrocyte sedimentation rate (ESR) and other parameters were measured by routine procedures. The GFR was measured by means of ^{51}Cr-EDTA clearance [5].

Statistics

Differences between values obtained at high and low disease activity were statistically evaluated by Wilcoxon's matched pairs signed rank test and analyses of correlation were performed by the Spearman rank correlation test [24].

Results

With few exceptions the levels of C1q, C1s, C4, C3 and C5 were lower in exacerbations than in remission (fig. 1). For C1s and C5 the values varied between normal and high. C1r-C1s-C1 IA were consistently present in raised amounts (fig. 2).

2 patients showed immunochemically low C2 in serum and plasma during active disease (fig. 3). By the functional assay, C2 levels were lower in serum than in plasma, with marked differences being noted in some patients. The functionally determined C2 in serum followed the disease activity more consistently than immunochemical C2 determinations.

During active disease only 2 patients (I and II) showed a normal lytic pattern in the screening test for the classical pathway. 6 patients gave a normal lytic reaction during low disease activity.

The alternative pathway screening test was normal in all patients when the disease activity was low. During active disease 1 patient (IV) demonstrated impaired lysis in the assay. This patient also showed a very low B level and the presence of Bb fragments in small concentrations in plasma (2% of reference). In the other patients the B levels were essentially normal by functional and immunochemical measurement and appeared to vary independently of disease activity (Fig. 1). Bb fragments in plasma were not found. There were only minor discrepancies between the immunochemical and the functional factor B values.

The levels of P were essentially within the normal

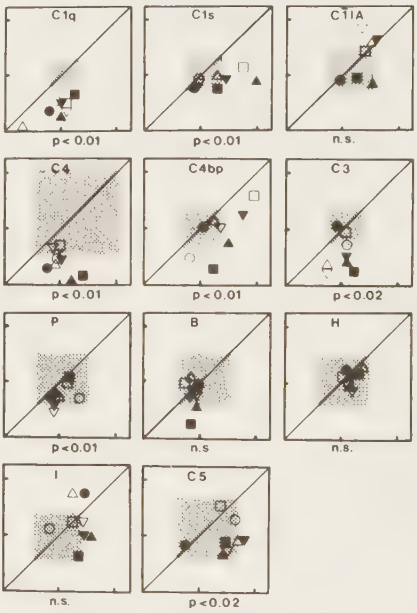


Fig. 1. Immunochemically determined levels of C1q, C1s, C1 IA, C4, C3, P, B, I and C5 at high (vertical axis) and low (horizontal axis) disease activity in 8 patients with SLE. The reference areas are shaded. Values are given in percent of normal with the 100% and 200% values indicated on each axis. Individual symbols are used for each of the patients: I = ○; II = ●; III = □; IV = ■; V = ▽; VI = △; VII = ▼; VIII = ▲. The level of significance, when values at high and low disease activity were compared, is given for each component (n.s. = not significant).

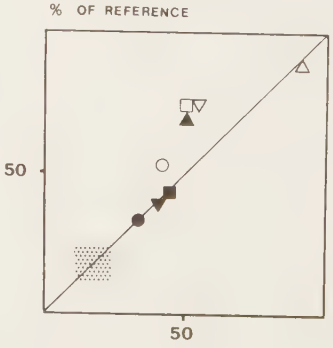


Fig. 2. C1r-C1s-C1 IA in the 8 SLE patients at high (vertical axis) and at low (horizontal axis) disease activity. The normal range is shaded. Patient symbols are used as in figure 1.

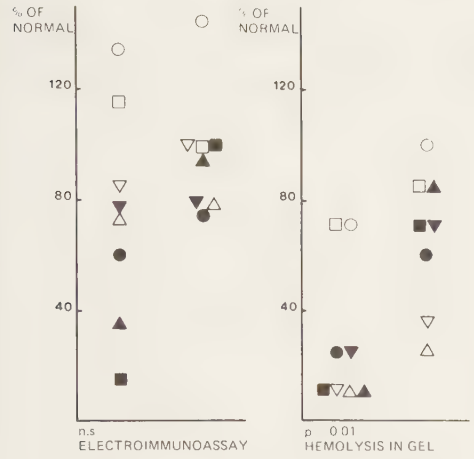


Fig. 3. C2 estimated immunochemically by electroimmunoassay and functionally by hemolysis in gel in active (left) and in inactive (right) SLE. Values are given in percent of normal. The reference areas were 112 ± 21% (SD) for the immunochemical test and 98 ± 27% (SD) for the functional test. For each patient the same symbol as in figure 1 is used.

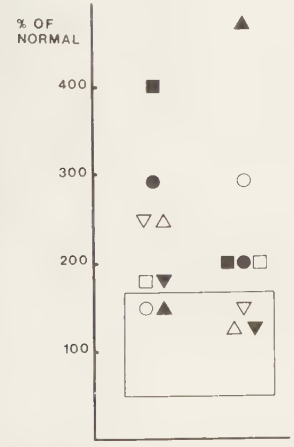


Fig. 4. Factor D determined functionally by hemolysis in gel in 8 SLE patients in active (left) and in inactive (right) disease. Values are given in percent of normal. The observed range in 50 normal adults is shown by a box. For each patient the same symbol as in figure 1 is used.

range but were lower in active than in inactive SLE (fig. 1).

The D activity in serum and plasma was markedly raised in most patients (fig. 4). The highest levels were found in 2 patients with reduced renal function (IV and VIII), but there was no clear-cut correlation between factor D concentrations and the GFR. The variation of factor D did not reflect changes in disease activity.

The levels of C1 IA, I and H did not vary consistently according to disease activity but C4bp was significantly lower in exacerbation than in remission (fig. 1). The patient with evidence for factor B activation (IV) showed lower I concentrations than other patients. In this patient, C4bp and C3 were markedly low during active disease while P and factor H were within the normal range with values similar to those found in remission.

Circulating immune complexes detected by C1qBA and C1qSP were present in all the patients. The range of the C1qBA values was 500–3,200 μg/ml in active disease and 270–9,000 μg/ml in inactive disease. The range of the C1qSP values was 25–1,550 μg/ml in exacerbation and 20–525 μg/ml in remission. As measured by C1qSP, the immune complex levels were significantly higher during active than during inactive SLE ($p < 0.01$). Such a relationship was not found for the C1qBA.

In active disease a positive correlation was found between the levels of C1r-C1s-C1 IA and immune complexes estimated by C1qSP ($r = 0.68$, $p < 0.05$) but not by C1qBA ($r = -0.07$). In remission C1r-C1s-C1 IA and immune complexes were not correlated (C1qSP, $r = 0.31$; C1qBA, $r = 0.05$).

Additional laboratory information is summarized in table II. The values of IgG varied according to disease activity while such an association was not found for hemoglobin, lymphocyte counts in peripheral blood and ESR. 6 of the patients (I, III–VI and VIII) had a positive *Crithidia luciliae* test, all with higher titers in active than in inactive disease. 1 patient (VII) with nephritis showed a negative *C. luciliae* test.

The 3 patients with increased CRP levels (I, III, VI) presented with serosisits and arthritis and 1 of them had a complicating bronchopneumonia (III). Pleural effusions from patients No. I and No. VI were bacteriologically sterile. The bronchopneumonia in No. III responded promptly to treatment with antibiotics.

Discussion

The present study concerned a small group of clinically well-defined SLE patients, who were studied at

high and low disease activity. Evidence of glomerulonephritis was present in most of the patients and with few exceptions, antibodies to native DNA were found during active disease. Raised CRP levels were recorded in 3 patients. Only 1 of these showed a bacterial infection indicating that the CRP response in SLE is not restricted to patients with superimposed infections [18].

$C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA in serum signify C1 activation [14] and have been demonstrated in raised amounts in rheumatoid arthritis [4] and in other diseases with immunological implications [9, 12, 26]. The SLE patients in the present study showed markedly high concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA with values clearly exceeding those previously found in active rheumatoid arthritis [4]. The concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA were similar in active and in inactive disease. During active disease the C1 activation was associated with low C1q, C4 and functional C2 and with decreased C3 levels consistent with sequential activation of the classical pathway. In contrast, the classical pathway component levels were essentially normal in periods of remission, indicating that efficient C activation did not proceed beyond C1. The significance of these findings in relationship to pathogenetic mechanisms and prognosis in SLE will require further evaluation.

As reported by *Abrass* et al. [2] circulating immune complexes as measured by C1qSP, but not by C1qBA, reflected changes in disease activity. It is not necessary to assume that circulating immune complexes accounted for the C1 activation in the patients, since tissue-bound complexes and other mechanisms might contribute to C1 activation with formation of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA [14]. On the other hand, $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA in active SLE were positively correlated with the immunoglobulin-containing complexes detected by C1q SP, indicating that at least part of the C1q-binding substances found could be responsible for the C1 activation.

Discrepancies were noted between serum and plasma values of functional C2 and more pronounced between functional and immunochemical C2 values. As the antiserum used detects native as well as cleaved C2 it seems likely that C2 fragments were present in some SLE plasma samples and that additional C2 inactivation occurred in the sera. In contrast, there were no discrepancies between functional and immunochemical factor B values.

The variation of P within the normal range, with relatively decreased values during active disease, could reflect alternative pathway activation, but there was no associated evidence of factor B, H and I consumption or of factor B cleavage. The data did not support a critical role of the alternative pathway in SLE. Also in larger series of SLE patients, the concentrations of factor B and P were found to be essentially normal [27].

1 patient (IV) with disseminated intravascular coagulation and pronounced hypocomplementemia during active SLE showed low factor B, Bb fragments and a relative decrease of factor I. In this patient the concentrations of P and H were similar in active and in inactive disease. The decrease of factor I could have been related to the low concentrations of C4bp. Judging from serum levels and other findings [33], C4bp might be consumed in the course of classical pathway activation.

The hemolytic diffusion in gel assay used is probably specific for factor D [17]. The high factor D levels in the SLE patients were confirmed with an enzyme amplified electroimmunoassay (to be published). The serum levels of other low molecular weight proteins such as β_2-microglobulin [10] are markedly influenced by the GFR and by tubular dysfunction. In analogy, the very high factor D levels in patients No. IV and No. VIII could be due to reduced GFR. However, high factor D levels were also found in patients without evidence of renal involvement.

Acknowledgement

This work was supported by grants from the Swedish Medical Research Council (B83-16X-68), the Medical Faculty, University of Lund, Konung Gustav V:s 80-årsfond, Riksförbundet mot reumatism, Greta och Johan Kocks Stiftelser, Föreningen för bistånd åt vanföra i Skåne, and Alfred Österlunds Stiftelse.

References

1 Aarden, L.A.; Groot, E.R. de; Feltkamp, T.E.W.: *Crithidia lucilliae*, a simple substrate for the determination of anti-dsDNA with the immunofluorescence technique. Ann. N.Y. Acad. Sci. *254:* 505–515 (1975).

2 Abrass, C.K.; Nies, K.M.; Louie, J.S.; Border, W.A.; Glassock, R.J.: Correlation and predictive accuracy of circulating immune complexes with disease activity in patients with systemic lupus erythematosus. Arthritis Rheum. *23:* 273–282 (1980).

3 Aguado, M.T.; Perrin, L.H.; Ramirez, R.; Miescher, P.A.; Lambert, P.H.: Evaluation of alternative pathway and factor B hemolytic activities in patients with systemic lupus erythematosus: correlations with the alternative pathway of regulatory proteins. Clin. exp. Immunol. *42:* 495–505 (1980).

4 Berglund, K.; Laurell, A.B.; Nived, O.; Sjöholm, A.G.; Sturfelt, G.: Complement activation, circulating C1q binding substances and inflammatory activity in rheumatoid arthritis: relation and changes on suppression of inflammation. J. clin. lab. Immunol. *4:* 7–14 (1980).

5 Bröchner-Mortensen, J.: A simple method for the determination of glomerular filtration rate. Scand. J. clin. Lab. Invest. *30:* 271–274 (1972).

6 Cohen, A.S.; Reynolds, W.E.; Franklin, C.E.; Kulka, E.C.; Ropes, M.W.; Schulman, L.E.; Wallace, S.L.: Preliminary criteria for the classification of systemic lupus erythematosus. Bull. rheum. Dis. *21:* 643–648 (1971).

7 Gewurz, H.; Pickering, R.J.; Mergenhagen, S.E.; Good, R.A.: The complement profile in acute glomerulonephritis, systemic lupus erythematosus and hypocomplementemic chronic glomerulonephritis: contrasts and experimental correlations. Int. Archs Allergy appl. Immun. *34:* 556–570 (1968).

8 Hughes, G.R.V.: Connective tissue diseases, p. 3 (Blackwell Scientific Publications, Oxford 1977).

9 Johnson, U.; Kamme, C.; Laurell, A.B.; Nilsson, N.: C1 subcomponents in acute pneumococcal otitis media in children. Acta pathol. microbiol. scand., C, Immunol. *75:* 10–16 (1977).

10 Karlsson, F.A.; Wibell, L.; Evrin, P.E.: β_2-microglobulin in clinical medicine. Scand. J. clin. Lab. Invest. *40:* suppl. 154, pp. 27–37 (1980).

11 Lachmann, P.J.; Hobart, M.J.: Complement technology; in Weir, Handbook of experimental immunology, vol. I, Immunochemistry, chapt. 5A, p. 1 (Blackwell Scientific Publications, Oxford 1978).

12 Laurell, A.B.; Mårtensson, U.; Sjöholm, A.: Studies on C1 subcomponents in chronic urticaria and angioedema. Int. Archs Allergy appl. Immun. *54:* 434–442 (1977).

13 Laurell, A.B.; Mårtensson, U.; Sjöholm, A.: The development of simple tests for C1q, C1r, C1s, C2 and properdin; in Opferkuch, Rother, Schultz, Clinical aspects of the complement system. Int. Symp., Bochum 1976, p. 12 (Thieme, Stuttgart 1978).

14 Laurell, A.B.; Mårtensson, U.; Johnson, U.; Sjöholm, A.G.: Formation of complexes composed of C1r, C1s and C1 inactivator in human serum on activation of C1. Acta pathol. microbiol. scand., C, Immunol. *86:* 299–306 (1978).

15 Laurell, A.B.; Mårtensson, U.; Sjöholm, A.G.: Quantitation of C1r-C1s-C1 inactivator complexes by electroimmunoassay. Acta pathol. microbiol. scand., C, Immunol. *87:* 79–81 (1979).

16 Laurell, C.B.: Electroimmunoassay. Scand. J. clin. Lab. Invest., suppl. *124:* 21–37 (1972).

17 Martin, A.; Lachmann, P.J.; Halbwachs, L.; Hobart, M.J.: Hemolytic diffusion plate assays for factors B and D of the alternative pathway of complement activation. Immunochemistry *13:* 317–324 (1976).

18 Pepys, M.B.: Serum C-reactive protein, serum amyloid P-component and serum amyloid A protein in autoimmune disease; in Holborow, Clinics in immunology and allergy, vol. 1, pp. 77–101 (1981).

19 Perrin, L.H.; Lambert, P.H.; Miescher, P.A.: Properdin levels in systemic lupus erythematosus and membranoproliferative glomerulonephritis. Clin. exp. Immunol. *16:* 575–581 (1974).

20 Perrin, L.H.; Lambert, P.H.; Miescher, P.A.: Complement breakdown products in plasma from patients with systemic lupus erythematosus and patients with membranoproliferative and other glomerulonephritis. J. clin. Invest. *56:* 165–176 (1975).

21 Ruddy, S.; Austen, K.F.: Natural control mechanisms of the complement system; in Ingram, Proc. Int. Symp. Biological Activities of Complement, pp. 13–26 (Karger, Basel 1972).

22 Schur, P.: Complement in lupus. Clin. rheum. Dis. *1:* 519–543 (1975).

23 Scullion, M.; Balint, G.; Whaley, K.: Evaluation of the C1q solid-phase binding assay for immune complexes. A clinical and laboratory study. J. clin. lab. Immunol. *2:* 15–22 (1979).

24 Siegel, S.: Nonparametric statistics for the behavioral sciences (McGraw Hill, New York 1956).

25 Sjöholm, A.G.: Complement components in normal serum and plasma quantitated by electroimmunoassay. Scand. J. Immunol. *4:* 25–30 (1975).

26 Sjöholm, A.G.: Complement components and complement activation in acute post-streptococcal glomerulonephritis (AGN). Int. Archs Allergy appl. Immun. *58:* 274–284 (1979).

27 Svensson, B.: Occurrence of deficient monocyte yeast cell phagocytosis in presence of rheumatic sera. Scand. J. Rheumatol., suppl. *31:* 21–27 (1980).

28 Truedsson, L.; Sjöholm, A.G.; Laurell, A.B.: Screening for deficiences in the classical and alternative pathways of the complement by hemolysis in gel. Acta pathol. microbiol. scand., C, Immunol. *89:* 161–166 (1981).

29 Ziegler, J.B.; Rosen, F.S.; Alper, C.A.; Grupe, W.; Lepow, I.H.: Metabolism of properdin in normal subjects and in patients with renal disease. J. clin. Invest. *56:* 761–767 (1975).

30 Zubler, R.H.; Lange, G.; Lambert, P.H.; Miescher, P.A.: Detection of immune complexes in unheated sera by a modified 125-I-C1q binding test. Effect of heating on the binding of C1q by immune complexes and application of the test to systemic lupus erythematosus. J. Immun. *116:* 232–235 (1976).

31 Whaley, K.; Schur, P.H.; Ruddy, S.: Relative importance of C3b inactivator and βIH globulin in the modulation of the properdin amplification loop in systemic lupus erythematosus. Clin. exp. Immunol. *36:* 408–414 (1979).

32 Wilson, M.R.; Arroyave, C.M.; Nakamura, R.; Vaughan, J.; Tan, E.M.: Activation of the alternative pathway of complement in systemic lupus erythematosus. Clin. exp. Immunol. *26:* 11–20 (1976).

33 Wyatt, R.J.; McAdams, A.J.; Forrestal, J.; Snyder, J.; West, C.D.: Glomerular deposition of complement-control proteins in acute and chronic glomerulonephritis. Kidney int. *16:* 505–512 (1979).

Received: May 24, 1982

Correspondence to: Dr. G. Sturfelt,
Department of Rheumatology,
University Hospital,
S-221 85 Lund (Sweden)

II

CLEAVAGE OF C2 IN PATHOLOGICAL SERUM AND PLASMA STUDIED BY CROSSED IMMUNOELECTROPHORESIS

A.G. Sjöholm [1] & G. Sturfelt [2]

[1] Department of Immunology, Institute of Medical Microbiology, and [2] Department of Rheumatology, University of Lund, Lund, Sweden.

Sjöholm A.G. & Sturfelt G.: Cleavage of C2 in pathological serum and plasma studied by crossed immunoelectrophoresis.

ABSTRACT

Cleaved C2, C2a, was demonstrated by crossed immunoelectrophoresis in serum and EDTA plasma from patients with active systemic lupus erythematosus (SLE), patients in the early phase of acute poststreptococcal glomerulonephritis (AGN), and from two males with congenital deficiency of $C\bar{1}$ inactivator, one of whom had symptoms of hereditary angioedema. C2a was not found in normal serum and plasma. C2 was more stable in plasma than in serum with regard to the effects of storage in room temperature and of repeated freezing and thawing. C2a concentrations were higher in serum than in plasma samples from SLE and AGN patients. Expressed in per cent of the total C2 protein, C2a was inversely correlated (r=-0.91) with the C2 hemolytic activity in the samples, which explains discrepancies between the results of immunochemical and functional assays, when used to measure C2 concentrations in disease.

Key words: Complement, C2 activation, C2 fragments, crossed immunoelectrophoresis.

INTRODUCTION

C2 is a natural substrate of C1 esterase ($C\bar{1}s$), which cleaves the molecule into C2a and C2b with the approximate molecular weights of 74 kD and 34 kD, respectively (7,18, 20). C4 is also activated by $C\bar{1}s$, and the major C4 fragment, C4b, forms a Mg^{2+} dependent complex with native C2 (20). The affinity between C2 and C4b is probably due to properties of the C2b domain of C2 (8,18). Cleavage of C2 in the C4b,C2 complex gives rise to classical pathway C3 convertase (C4b,2a), the enzymatic activity

of which resides in the C2a fragment (2,9). The C4b,2a enzyme decays rapidly with the dissociation of C2a (9,26). With some conditions, C2b has been demonstrated both in the enzymatically active complex and in decayed convertase in association with C4b (9).

As shown by Nagasawa & Stroud (18), C2a and C2b carry distinct antigenic determinants that can be recognized by antisera raised against native C2. Nagasawa & Stroud also reported that on immunoelectrophoresis, C2a appeared in a position anodal to native C2 in a purified system, while C2b migrated towards the cathode. In the present investigation, C2 in serum and EDTA plasma was studied by crossed immunoelectrophoresis. Samples from patients with either systemic lupus erythematosus (SLE), acute poststreptococcal glomerulonephritis (AGN), or hereditary angioedema (HAE), were all found to contain C2a, the concentrations of which could be expressed as a percentage of cleaved C2. Functional and immunochemical C2 determinations were also performed.

MATERIALS ANS METHODS

Serum and EDTA plasma. Serum and EDTA plasma samples from 20 healthy donors and from patients were stored in aliquots at -80 ^{o}C. Serum from a healthy male with homozygous C2 deficiency was available in the laboratory.

Patients. Four patients with active SLE (27), and two patients with AGN (23) with typical complement profiles were studied. From the AGN patients, samples were drawn during the first week after the onset of AGN, and 4-6 months later. Two males with congenital $C\bar{1}$ inactivator deficiency, one with symptoms of HAE and the other an asymptomatic child with the HAE trait, were also investigated.

C2 preparations and rabbit anti-C2 antibody. Partially purified C2 was obtained as described previously (11), and was further purified (20) on hydroxylapatite (Bio-Gel HT, Bio-Rad Laboratories, USA). Early bleedings from a rabbit given purified C2 subcutaneously in Freunds complete adjuvant, and a second dose of partially purified C2 intravenously a week later, provided antiserum that was monospecific for C2 in routine immunochemical tests. Other anti-C2 antisera were rendered monospecific by appropriate absorption. The IgG fraction of anti-C2 was used (25). In more recent experiments, C2 was purified according to the general procedure given by Kerr (8), though hydroxylapatite was used to separate C2 from factor B.

Purified $C\bar{1}s$ was isolated as described previously and the esterolytic activity given in U/ml (24). The $C\bar{1}s$ was added to serum, EDTA plasma or C2 preparations at 100 U/ml.

Heat aggregated human IgG (AHG) was prepared as described previously (13). Sera were incubated at 37°C with AHG at final concentrations ranging between 0.1 and 1 mg/ml. After incubation, EDTA (10 mmol/l) was added to the sera.

Stability of C2 in serum and EDTA plasma. Serum and EDTA plasma from five healthy individuals were divided into aliquots that were either frozen immediately at -80°C or first kept at room temperature for 1, 3 or 5 days. Aliquots were also frozen 5 times at -80°C and thawed by exposing them to room temperature for 1 hour. Some samples were incubated at 37°C for one hour before analysis.

Measurement of C2. C2 was measured immunochemically by electroimmunoassay as described previously (12), and functionally, using C2 deficient serum, by hemolytic titration (19) and by hemolysis in gel (10).

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed according to Ganrot (4), using barbital buffer, 75 mmol/l, pH 8.6, containing EDTA at 2 mmol/l, though EDTA was

omitted from the buffer in some experiments. In the second electrophoretic step, polyethylene glycol 6000 (KEBO AB, Stockholm, Sweden) at 5 % w/v was included in the agarose gel (Agarose (ME), Miles Laboratories Ltd, England). EDTA was usually added to all samples at 10 mmol/l before electrophoresis. Results were evaluated by planimetry, the proportion of C2a being expressed as a percentage of the area covered by precipitated C2 protein.

Immunoelectrophoresis (21) was performed with the same buffers as those used for crossed immunoelectrophoresis. Samples analyzed were applied in 5-10 µl volumes.

Statistics. Analyses of correlation were performed by the Spearman rank correlation test (22).

RESULTS

Normal EDTA plasma, or serum with EDTA added, gave a symmetrical C2 precipitate in the β_2-region on crossed immunoelectrophoresis (fig.1a). Preparations of functionally active C2 applied alone, or in C2-deficient EDTA serum, gave a similar β_2-precipitate.

With C1s added to serum or EDTA plasma, the mobility of the C2 protein shifted to the fast β_1-region (Fig. 1b). The fast β_1- precipitate was more weakly stained within its contour-line than was the β_2-precipitate. The C2 in normal serum with AHG added at 0.5 - 1 mg per ml had completely shifted to the fast β_1-region within 15 minutes of incubation at 37°C.

Normal sera, analyzed in the absence of EDTA during the first electrophoretic step, consistently gave a C2 precipitate in the fast β_1-region, sometimes combined with a minor β_2-precipitate.

In a few experiments, C2 was studied by immunoelectrophoresis. With native C2 precipitating in the β-region, some antisera gave

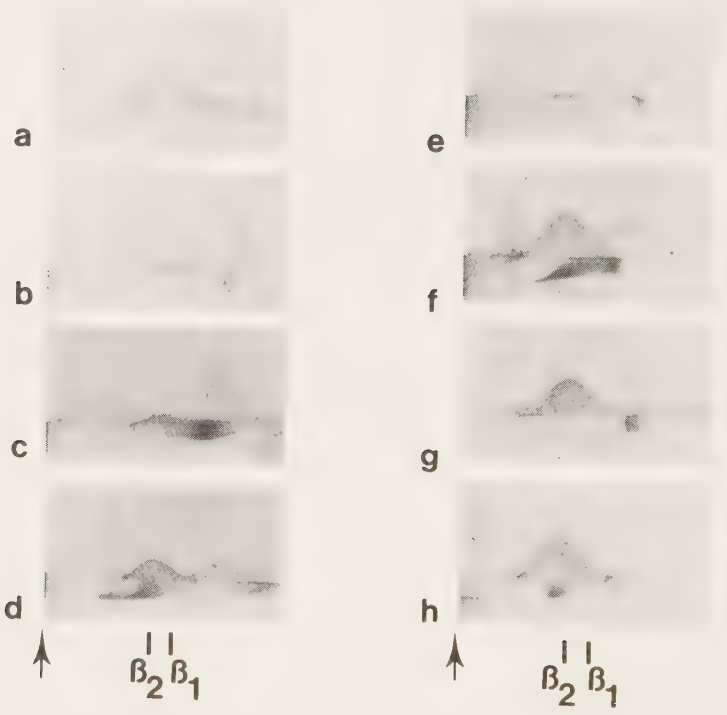


Fig. 1

Crossed immunoelectrophoresis of C2 in serum and EDTA plasma. EDTA (10 mmol/l) was added to all samples before electrophoresis.

a. Normal serum

b. Normal serum+ $C\bar{1}s$ (100 U/ml)

c. SLE serum

d. SLE plasma (on the same occasion as in c).

e. AGN serum (first week after onset of disease)

f. AGN plasma (on the same occasion as in e).

g. AGN plasma (reconvalescence)

h. AGN plasma (on the same occasion as in g).

Arrow indicates application site.

both an anodal and a more faint cathodal arc when $C\bar{1}s$ treated C2 preparations were examined. Judging from the results of Nagasawa & Stroud (18), the anodal C2 cleavage product here was C2a and the cathodal component C2b. C2b fragments were not detected in $C\bar{1}s$ treated sera with the conditions used.

Pathological serum and EDTA plasma samples were studied by crossed immunoelectrophoresis, EDTA being added to the sera before analysis. C2 in serum and plasma from the patient with HAE was completely converted to C2a. Samples from the asymptomatic child with the HAE trait showed partial C2 conversion with similar amounts of C2a in serum and plasma.

C2 was partially cleaved in samples from the four SLE-patients. The sera consistently contained more C2a than the plasma samples. The C2 patterns in serum and plasma from one of the patients are shown in Fig. 1,c & d.

C2a was also found in samples from the two AGN patients during the early phase of the disease. As with SLE, the cleavage of C2 was more pronounced in serum than in plasma. In samples obtained 3-6 months after the onset of symptoms, C2 cleavage was slight or undetectable. C2 patterns in serum and plasma from one of the patients are shown in Fig. 1, e-h.

Planimetrical evaluation of precipitates obtained by crossed immunoelectrophoresis provided values for C2a expressed as percentages of the C2 protein in the samples. The lower limit for reliable detection of C2a ranged from 5 to 10 per cent , partly dependent on the C2 concentrations present.

Incubation of normal serum and EDTA plasma at 37°C for up to one hour did not result in the generation of detectable amounts of C2a . After one day of storage at room temperature, normal sera showed trace amounts of C2a, while no C2a was found in plasma samples treated in the same way. After three days, the sera showed substantial C2 cleavage (30-40 per cent) and plasma samples contained C2a in small amounts (< 10 per cent). Freezing

and thawing five times gave rise to small amounts of C2a in normal sera, while the C2 remained intact in EDTA plasma.

In pathological samples C2 was measured functionally by hemolytic titration and by hemolysis in gel, results obtained with the two assays being closely correlated (r = 0.95). The C2 values were lower in serum than in plasma, whereas no differences between the serum and plasma C2 values were found, when C2 was measured by electroimmuoassay. In most pathological samples, the functional assays gave lower C2 values than the immunochemical test, a difference which was most apparent when C2 was determined in serum (Fig. 2). The C2a values in pathological serum and EDTA plasma samples showed an inverse relationship (r = - 0.91) to the C2 titres (Fig. 3). A similar relationship was found when C2a measured by crossed electrophoresis was compared with C2 measured by hemolysis in gel (r = - 0.85).

DISCUSSION

As previously found by Nagasawa & Stroud (18), when $C\bar{1}s$-treated C2 preparations were studied by immunoelectrophoresis, antiserum to C2 recognized two cleavage products. The predominant precipitate appeared in a position anodal to that of native C2 in the region, and evidently represented C2a, while C2b gave a more weakly-stained cathodal precipitate. Normal sera treated with $C\bar{1}s$ only produced the anodal C2 cleavage product. Possibly, C2b in serum was not detected due to complex formation with C4b (9,18).

Native C2, and C2a fragments were investigated in greater detail using crossed immunoelectrophoresis. As previously shown with C4, the C2 in normal sera was cleaved during electrophoresis, unless EDTA was added to prevent C1 activation (24). Treatment of serum with AHG at concentrations known to activate C1 (13),

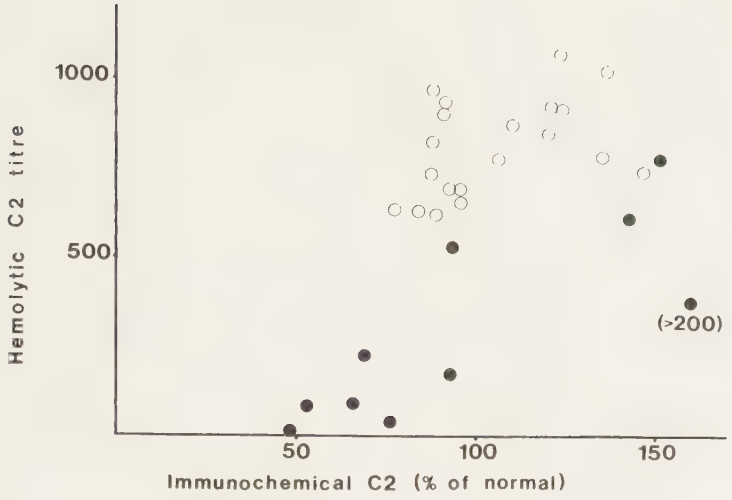


Fig. 2

Immunochemical C2 values (abscissa) and the C2 titres (ordinate) in normal sera (open symbols) and in the pathological sera investigated (solid symbols).

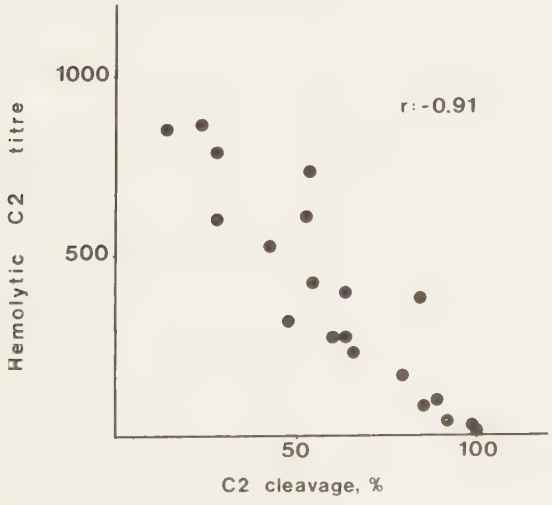


Fig. 3

Concentration of cleaved C2, C2a, in pathological serum and EDTA plasma compared with the titre of hemolytic C2 in the samples. The C2a values were obtained by planimetry of C2 patterns on crossed immunoelectrophoresis and were given in % of cleaved C2.

or with $\overline{C1}s$ resulted in complete conversion of C2 to C2a.

C2a, often in considerable amounts, was present in serum and EDTA plasma from patients with active SLE, AGN or with HAE. C1q binding immune complexes, and increased C1 activation have been reported in SLE (27) and in the early phase of AGN (23). Since C4 and C2, when activated by $\overline{C1}$, form the C3 convertase of the classical pathway, the present findings in SLE and AGN suggest that the C2 was cleaved in the course of activation of the classical pathway. In HAE, C1 is known to be activated due to partial deficiency of the $\overline{C1}$ inactivator, resulting in low C4 and C2 titres (3).

The demonstration of C2a in EDTA plasma suggests that circulating C2a is present in immune complex diseases such as AGN and SLE. The concentrations of C2a were consistently higher in the patient sera, probably owing to complement activation that had occurred after sampling. Concerning the presence of C2a in samples obtained from HAE patients, it should be borne in mind that $\overline{C1}s$ activity can be detected both in HAE serum and EDTA plasma (16).

The proportion of C2a was inversely correlated with the C2 hemolytic activity in pathological samples, which demonstrated the relationship between C2 cleavage and inactivation of functional C2. The findings explain discrepancies between functional and immunochemical C2 values previously observed in active SLE (27,28). Similarly in AGN, C2 titres have been reported to be markedly low (5), in contrast to the normal or moderately reduced C2 values determined immunochemically (23).

Quantitative assays have been described for the demonstration of C1 activation (6,14), and of C4 (17) and C3 fragments (1). Together with these, and other assays, measurement of C2a provides an interesting means of assessing classical pathway activation in disease. Comparisons that have been made between functional and immunochemical C2 determinations in various diseses suggest that C2 cleavage mainly occurs in conjunction

with other evidence of complement activation (15,27,28). The apparent stability of native C2 in EDTA plasma adds to the reliability of C2a determinations, when such samples are used. As shown here, crossed immunoelectrophoresis is useful in studying C2 cleavage. The development of less time-consuming assays for measurement of C2a in clinical laboratory work should be considered.

ACKNOWLEDGMENTS

This investigation was supported by grants from the Swedish Medical Research Council (B83-16X-68), the Medical Faculty, University of Lund, Konung Gustav V:s 80 års fond, Riksförbundet mot reumatism, Greta och Johan Kochs Stiftelser, Thorsten och Elsa Segerfalks Stiftelse, Alfred Österlunds Stiftelse, Förenade Liv, Ömsesidigt Gruppförsäkringsbolag, Stockholm and Kungliga Fysiografiska Sällskapet i Lund.

REFERENCES

1. Brandslund, I., Sierstad, H.C., Svehag, S.-E. & Teisner, B. : Double-decker rocket immunelectrophoresis for direct quantitation of complement C3 split products with C3d specificities in plasma. J. Immunol. Methods. 44 :63 - 71, 1981.

2. Cooper, N.R. : Enzymatic activity of the second component of complement. Biochemistry 14 : 4245 - 4251, 1975.

3. Frank, M.M., Gelfand, J.A. & Atkinson, J.P. : Hereditary angioedema: the clinical syndrome and its management. Ann. Intern. Med. 84 : 580 - 593, 1976.

4. Ganrot, P.O. : Crossed immunoelectrophoresis. Scand. J. clin.Lab. Invest. 29 , suppl 124: 39 - 47, 1972.

5. Gewurz, H., Pickering, R.J., Mergenhagen, S.E. & Good, R.A. : The complement profile in acute glomerulonephritis, systemic lupus erythematosus and hypocomplementemic chronic glomerulonephritis. Contrasts and experimental correlations. Int. Archs Allergy appl. Immun. 34 : 556 - 570, 1968.

6. Hack, C.E., Eerenberg-Belmer A.J.M., Hannema A.J. , Out T.A. & Aalberse R.C. : A $C\bar{1}$-inhibitor complex assay (INCA): a method to detect C1 activation in vitro and in vivo. J.Immunol. 127 : 1450 - 1453, 1981.

7. Kerr, M.A. & Porter R.R. : The purification and properties of the second component of human complement. Biochem. J. 171 : 99 - 107, 1978.

8. Kerr, M.A. : Limited proteolysis of complement components C2 and

factor B. Biochem. J. 183 :615 - 622, 1979.

9. Kerr, M.A. : The human complement system: assembly of the classical pathway C3 convertase. Biochem.J. 189 : 173 - 181, 1980.

10. Lachmann, P.J. & Hobart, M.J. : Complement technology. In: Weir (Ed.) Handbook of experimental immunology, vol. 1. Immunochemistry. Blackwell Scientific Publications, Oxford 1978. Chapt 5 A.

11. Laurell, A.-B., Mårtensson, U. & Sjöholm, A.G. : C1 subcomponent complexes in normal and pathological sera studied by crossed immunoelectrophoresis. Acta path. microbiol. scand. Sect.C, 84 : 455 - 464, 1976.

12. Laurell, A.-B., Mårtensson U. & Sjöholm, A.G. : The development of simple tests for C1q, C1r, C1s and C2, and the determination of properdin. In: Opferkuch, Rother & Schultz (Eds) Clinical aspects of the complement system. Thieme, Stuttgart 1978, pp 12 - 14.

13. Laurell, A.-B., Johnson, U., Mårtensson, U. & Sjöholm A.G. : Formation of complexes composed of $C\bar{1}r$, $C\bar{1}s$, and $C\bar{1}$ inactivator in human serum on activation of C1. Acta path. microbiol. scand. Sect. C, 86 : 299 - 306 ,1978.

14. Laurell, A.-B., Mårtensson, U. & Sjöholm, A.G. : Quantitation of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ inactivator complexes by electroimmunoassay. Acta path. microbiol. scand. Sect C, 87 : 79 - 81, 1979.

15. Lopez-Trascasa M., Prevot, D., Moisy, M., Blanc, C., Lagrue, G. & Sobel A.T. : Quantitation of C2 by rocket immunoelectrophoresis in 120 pathological sera. Path. Biol. 29 : 481 - 485, 1981.

16. Lundh, B., Laurell, A.-B., Wetterquist, H., White, T. & Granerus, G. : A case of hereditary angioneurotic oedema, successfully treated with ε-aminocaproic acid. Studies on C'1-esterase

inhibitor, C'1 activation, plasminogen level and histamine metabolism. Clin. exp. Immunol. 3 : 733 - 745, 1968.

17. Milgrom, H., Curd,J.G., Kaplan, R.A., Müller-Eberhard, H.J. & Vaughan, J.H. : Activation of the fourth component of complement (C4): Assessment by rocket immunoelectrophoresis and correlation with the metabolism of C4. J.Immunol. 124 : 2780 - 2785, 1980.

18. Nagasawa, S. & Stroud R.M. : Cleavage of C2 by $\overline{C1}$s into antigenically distinct fragments C2a and C2b: Demonstration of binding of C2b to C4b. Proc. Natl. Acad. Sci. USA 74 : 2998 - 3001, 1977.

19. Ngan, B.-Y., Gelfand, E.W. & Minta J.O. : A simple one-step hemolytic assay for C2 with C2 deficient human serum. J. Immunol. 118 : 736 - 741, 1977.

20. Polley, M.J., & Müller-Eberhard, H.J. : The second component of human complement : its isolation, fragmentation by C'1 esterase, and incorporation into C'3 convertase. J.Exp. Med. 128 :533 - 551, 1968.

21. Scheidegger, J.J. : Une micro-methode l'immunoelectrophorese. Int. Arch. Allergy. 7 103 - 110, 1955.

22. Siegel, S. : Nonparametric statistics for the behavioral sciences (McGraw Hill, New York 1956).

23. Sjöholm, A.G. : Complement components and complement activation in acute poststreptococcal glomerulonephritis. Int. Archs Allergy appl. Immun. 58 : 274 - 284, 1979.

24. Sjöholm, A.G. & Laurell, A.-B. : Conversion of the fourth complement component studied by crossed immunoelectrophoresis. Clin. exp. Immunol. 14 : 515 - 529, 1973.

25. Steinbuch, M & Audran, R. : The isolation of IgG from mammalian sera with the aid of caprylic acid. Archs biochem. biophys. 134 :

279 - 284, 1969.

26. Stroud R.M., Mayer, M.M., Miller, J.A. & McKenzie A.I. : C'2a, an inactive derivative of C'2 released during decay of EAC'4,2a. Immunochemistry 3 : 163 - 176, 1966.

27. Sturfelt, G., Sjöholm A.G. & Svensson B. : Complement components, C1 activation and disease activity in SLE. Int. Archs Allergy appl. Immun. 70 : 12 - 18, 1983.

28. Ueda A., Kusaba T. & Yanase T. : Determination of the second component of complement (C2) by electroimmunassay in sera from patients with systemic lupus erythematosus. Clin. exp. Immunol. 53 : 217 - 224.

COMPLEMENT COMPONENTS, COMPLEMENT ACTIVATION, AND ACUTE PHASE RESPONSE IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Gunnar Sturfelt [1] & Anders G. Sjöholm [2]

[1] [2] Department of Rheumatology and Department of Immunology, Institute of Medical Microbiology, Lund University of Lund, Lund

---

Correspondence to Gunnar Sturfelt, Department of Rheumatology, University Hospital , S-22185 Lund.

ABSTRACT

The investigation concerned 33 SLE patients assigned to three groups representing: mild SLE, more severe extra renal SLE and SLE with significant renal involvement. In patients with extra renal disease, the inflammatory plasma protein response was often pronounced during exacerbation, as evidenced by markedly increased concentrations of C-reactive protein (CRP), α_1-antichymotrypsin, α_1-antitrypsin and orosomucoid. CRP responses were rare in patients with renal involvement, despite the increased concentrations of other acute phase reactants in some of these patients. Superimposed bacterial infections were not clearly distinguished by raised CRP concentrations. The classical pathway of complement (C) was activated in all patients during exacerbation, as indicated by increased concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ inactivator complexes and C2a fragments. C1 and C2 activation and probably also C3 activation varied according to the amounts of circulating C1q binding immune complexes, as measured by solid phase assay. Manifest hypocomplementemia was usually associated with glomerulonephritis. Participation of C components in the inflammatory plasma protein response apparently counteracted the development of hypocomplementemia in many patients with extra-renal SLE. Circulating C3d was detected in all patients during exacerbation of renal disease, and in most patients with severe extra-renal manifestations. Inverse relationships were found between immunochemical C2 concentrations and the percentage of cleaved C2, and between C3 and C3d. There was no appreciable consumption of factors B and D and properdin of the alternative pathway in the patients. High concentrations of factor D, a low molecular weight protein, were exclusively found in patients with renal involvement and could be ascribed to retention due to reduced glomerular filtration.

III

INTRODUCTION

Complement (C) activation, elicited by immune complexes, is a principal mechanism of inflammation in systemic lupus erythematosus (SLE) (22). C is known to be consumed in the disease, and hypocomplementemia with low concentrations of components such as C3 and C4 may be of diagnostic value in SLE (17). It must be borne in mind, however, that low concentrations of C components in SLE are sometimes genetically determined (21). Conversely, many C components behave like acute phase reactants in the inflammatory plasma protein response (23), whereby increased synthesis may balance C breakdown and counteract development of manifest hypocomplementemia. With the important exception of C-reactive protein (CRP), acute phase reactants have not been systematically investigated in SLE (19).

The present investigation concerned relationships between C component levels, C activation and the inflammatory plasma protein response in SLE. Concentrations of several C components were determined, and C activation further assessed by measuring complexes between $C\bar{1}$ inactivator and the C1 proteases $C\bar{1}r$ and $C\bar{1}s$ (12) and by studies of C2 and C3 fragments. The inflammatory plasma protein response was documented by determinations of CRP, α_1-antichymotrypsin, orosomucoid and α_1-antitrypsin. C1q binding immune complexes were measured by solid phase assay (24).

We recently found pronounced C1 activation in a small group of SLE patients, most of whom had glomerulonephritis and circulating antibodies to native DNA (nDNA) (29). Markedly high concentrations of the low molecular weight protein factor D were also recorded, attributable rather to retention due to reduced glomerular filtration than to increased synthesis (30).

The present investigation covered a broader range of SLE manifestations, studied in patients within equally sized groups

representing different aspects of the SLE syndrome. Investigations were carried out during exacerbations, during periods of low disease activity and also during superimposed infections.

MATERIALS AND METHODS

Patients. In 1980 a standardized SLE control program, now comprising more than 100 patients, was initiated at the Department of Rheumatology, University Hospital of Lund. The basis of SLE diagnosis was the presence of typical multisystemic disease, and the absence of features incompatible with the disease. Clinical and laboratory investigations were carried out at regular intervals of two months (6 - 8 weeks), and as appropriate to the course of the disease.

The present study concerned 33 patients all with varying disease activity as manifested in at least one exacerbation during an observation period of 10 - 30 months. This provided, the 11 patients in each of the three groups, as defined below, entered the study consecutively. All fulfilled more than 4 of both the 1971 and 1982 American Rheumatism Association classification criteria for SLE (6, 32).

Patients were assigned to groups according to the clinical manifestations that developed during the observation period, minor and major SLE manifestations being distinguished essentially as defined by Lightfoot and Hughes (16). Group I consisted of 11 women with mild extra-renal disease, and groups II and III of 3 men and 8 women in each, all with major manifestations. Group II patients showed extra-renal symptoms only and in group III evidence of active renal disease was found in all patients. Age on entry to the study varied between 16 and 60 years, with a median of 38 years in groups I and II, and of 32 years in group III. Duration of disease from the time of

diagnosis was 0 - 22 years, with a median of 2 years in each group.

All patients were ANA (anti-nuclear antibody) positive by indirect immunofluorescence on at least one occasion. Precipitating antibodies (31) to nuclear ribonucleoprotein (RNP) were detected in 18% of group I, and in 27% of the group II and of III and precipitating antibodies to Sm antigen in 18% of each group. Rabbit thymus (Extractable nuclear antigen, Pel-Freez Biologicals, Rogers, AR, USA) was used as the antigen source in immunodiffusion tests, and the specificity of antibodies was examined using ANA reference anti-RNP and anti-Sm sera (AF-CDC ANA Reference Lab., Atlanta, GA, USA). During active disease anti-nDNA antibodies (1) were detected in 36% of group I, in 64% of group II and in 82% of group III. Rheumatoid factors (Waaler-Rose test), present in 27% of groups I and II, were not found at all in group III patients.

Clinical manifestations and criteria of disease activity.
Clinical features of the three patient groups are summarized in Table I. With few exceptions, minor manifestations were restricted to the skin and to the musculosceletal system. Major manifestations were often combined with minor symptoms.

Pleuropericarditis was defined as pain accompanied by abnormal X-ray and/or EKG changes, and ultracardiogram findings consistent with the diagnosis. Exudates were examined by culture, glucose analysis and leukocyte counts.

For the evaluation of neuropsychiatric manifestations, trained physicians at the Neurological and Psychiatric Departments, University Hospital, Lund were consulted. Investigations included computerized tomography, cerebral blood flow measurement (Xe_{133}) and routine analysis of the cerebrospinal fluid.

The clinical evaluation of renal symptoms, and the treatment of nephritic patients were made in consultation with Dr H. Thysell,

Table I

Clinical manifestations observed during the study. Group I = patients with only minor manifestations, group II = patients with major manifestations but no evidence of renal involvement, group III = patients with active lupus nephritis.

Group I (n = 11)	No of patients
Arthritis	11
Cutaneous manifestations	7
Fever	7
Raynaud's phenomenon	2
Myositis	1
Leucocytopenia	4
Group 11 (n = 11)	
Pleuropericarditis	8
CNS-manifestations	2
Vasculitis with cutaneous ulcers	1
Fibrosing alveolitis	1
Pulmonary embolism	1
Pancreatitis	1
Leucocytopenia	4
Minor manifestations	11
Group III (n = 11)	
Glomerulonephritis	11
CNS-manifestations	2
Cardiomyopathy	1
Pulmonary embolism	1
Vasculitis with cutaneous ulcers	1
Leucocytopenia	4
Thrombocytopenia	1
Minor manifestations	11

Department of Nephrology, University Hospital, Lund.

There was no evidence of active renal involvement in group I and II patients during the course of the study, as judged by urinalysis and measurement of S-creatinine and blood pressure. One patient in group II had one episode of microscopic hematuria but further investigations provided no evidence of glomerulonephritis. One patient belonging to group I, in whom glomerulonephritis had been verified by renal biopsy 3 years earlier (unclassifiable glomerulonephritis) now had only minor manifestations, a normal glomerular filtration rate and no signs of active renal disease. Active renal involvement was present in all group III patients as evidenced by urinalysis and repeated measurement of renal function (S-creatinine and glomerular filtration rate determined by CrEDTA clearance (4)).

Renal disease was documented by biopsy in 10 of the group III patients. Light microscopy (Dr. C. Brun, Department of Clinical Chemistry , Kommunehospitalet, Copenhagen) showed proliferative glomerulonephritis in 5 patients, mesangioproliferative in 1, epimembranous in 1, and unclassifiable glomerulonephritis in 3 patients.

By definition, minor manifestations could be controlled by antimalarial drugs or Prednisolone in low dosage (<20 mg/day), or a combination of both. Patients with major manifestations were always treated with Prednisolone at more than 20 mg/day and were hospitalized during flares. Three patients in group II and 9 in group III were treated with cytostatic drugs. In 6 of the group III patients plasmapheresis was performed during short periods (1 - 2 weeks) as an adjunct to immunosuppression.

The investigations reported here were made during exacerbation, at the point of maximal disease activity before treatment was started, and during quiescent disease (the time of maximal clinical improvement and clinical stability). The first exacerbation was selected for study in patients with more than one flare during the observation period. The interval between

the two occasions selected for investigation varied from 4 to 12 months. In addition, samples obtained during superimposed infections were also studied.

Differential diagnosis against infection. In all patients with fever and/or major manifestations, the investigations included cultures from blood, urine and nasopharynx and serological screening for viral infections, additional investigations being performed as appropriate to the clinical picture.

Serum and plasma. Blood sampling was performed in conjunction with all investigations made according to the SLE control program. After venesection, clotting was allowed to procede for 1 h at +20°C and therafter for 1 h at +4°C. Blood collected in EDTA at 5 mmol/l was handled in the same way. After centrifugation, serum and EDTA plasma were frozen in aliquots and stored at -80°C until analysed.

Complement assays. C1q, C1s, C2, C4, C3, factor B (B), properdin (P) and $C\bar{1}$ inactivator ($C\bar{1}$ IA) were determined by electroimmunoassay (10,27), and factor D (D) by enzyme amplified electroimmunoassay (33). Values were given as percentages of the concentrations in a reference serum or EDTA plasma pool said to contain each component at 100%. The reference areas (95% confidence intervals) were based on values in 100 healthy adults.

C1 subcomponent complexes were studied by crossed immunoelectrophoresis (9). Complexes containing $C\bar{1}r$, $C\bar{1}s$ and $C\bar{1}$ inactivator ($C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA) in serum were measured by electroimmunoassay with values given in per cent of the $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA in a reference serum pool treated with heat-aggregated IgG (12). In normal sera the concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA was between 11 and 25% of that in the reference.

C3d was measured in plasma with "double-decker" electroimmunoassay (3) as modified by Johnson (8). Polyethylene glycol (PEG 6000) was not used for the pretreatment of samples

but was added (1% W/v) to the anti-C3d containing gel. C3d was given as a percentage of the C3d in a normal serum pool treated with inulin (100 mg/ml, 37°C, 120 min). No C3d was detected in plasma samples from 20 healthy blood donors. The lower limit for detection of C3d was about 6 %.

C2 cleavage was studied by crossed immunoelectrophoresis (28). Following planimetric evaluation , the proportion of cleaved C2 (C2a), was expressed as a percentage of the area covered by precipitated C2 protein. No C2 cleavage was detected in plasma samples from 20 healthy blood donors. The lower limit for reliable detection of C2a was between 5% and 10%, depending on the C2 content in the samples.

Acute phase proteins. CRP, α_1-antitrypsin, α_1-antichymotrypsin and orosomucoid were measured by electroimmunoassay (13). CRP values were given in mg/l and other acute phase proteins in per cent of the concentration in a human reference serum (Seronorm-R Protein, Nyegaard & Co, Oslo, Norway). The anti-sera towards α_1-antitrypsin and α_1-antichymotrypsin were kindly provided by Prof. C.-B. Laurell, Dept. of Clin. Chemistry, Malmö General Hospital.

Circulating immune complexes. Circulating immune complexes were determined by solid phase C1q binding assay (C1qSP) essentially as described by Scullion et al., (24) but slightly modified (29), the values being expressed as equivalents of aggregated human IgG (AHG) in mg/l. Control sera yielded values lower than 10 mg/l.

Statistics. Differences between values obtained during high and during low disease activity were statistically evaluated by the Wilcoxon matched pairs signed rank test (25). Correlations were analysed using the Spearman rank correlation test (25). Differences between groups of independent values were calculated by the Mann-Whitney U test (25).

TABLE II

Complement component levels in 33 SLE patients during exacerbation and remission of disease activity. Group I = 11 patients with mild SLE, group II = 11 patients with severe extra renal manifestations, and group III = 11 patients with lupus glomerulonephritis. Values were given in per cent of the normal.

	Exacerbation		Number of patients with abnormal values		Remission		Number of patients with abnormal values	
	median	range	low	high	median	range	low	high
Group I								
C1q	125	19 - 185	1	5	114	61 - 143	1	2
C1s	166	94 - 189	0	2	120	97 - 166	0	1
C2	103	43 - 181	2	1	100	38 - 148	3	0
C4	64	17 - 171	4	0	75	15 - 174	4	0
C3	87	33 - 150	2	2	89	36 - 115	1	0
P	109	74 - 124	0	0	108	81 - 149	0	0
B	117	77 - 193	0	3	97	77 - 150	0	0
D	104	68 - 122	0	0	100	67 - 123	0	0
Group II								
C1q	113	<5 - 193	5	4	105	87 - 194	0	2
C1s	127	89 - 151	0	1	111	97 - 162	0	3
[1]C2	140	102 - 197	0	3	129	85 - 149	0	0
C4	75	33 - 157	2	0	95	40 - 130	1	0
C3	91	33 - 199	4	2	95	70 - 130	0	0
P	87	46 - 182	1	1	97	69 - 128	0	0
B	135	98 - 196	0	4	105	68 - 136	0	0
D	93	66 - 129	0	0	97	85 - 127	0	0
Group III								
C1q	31	<5 - 136	9	1	87	5 - 145	2	1
C1s	93	34 - 143	3	2	99	40 - 149	1	1
C2	73	40 - 156	6	0	71	47 - 131	7	0
C4	35	5 - 92	9	0	48	15 - 115	8	0
C3	57	23 - 103	8	0	71	30 - 96	5	0
P	105	76 - 122	0	0	97	41 - 126	2	0
B	104	72 - 157	0	1	96	63 - 141	0	0
D	210	100 - 668	0	9	179	103 - 469	0	8

Reference areas: C1q 78 - 130; C1s 79 - 139; C2 75 - 163; C4 53 - 207; C3 70 - 136; P 57 - 153; B 59 - 154; D 75 - 143.

[1]One patient with C2 homozygous deficiency excluded.

RESULTS

Complement components. (Table II) Concentrations of C1q, C4, C2 and C3 were lower in patients with active glomerulonephritis (group III) than in patients with minor symptoms (group I), or with major extra renal manifestations (group II). In groups I and II, the values varied within a wide range. Several patients in groups I and II had increased concentrations of C1q during active disease. Very low C1q (<5%) was found in one group II patient who entered the study in a bad general condition with high fever, serositis, neuropsychiatric and skin and joint manifestations but without evidence of renal involvement. Another group II patient with homozygous C2 deficiency had normal or high concentrations of C1q, C1s and C3. Factor B concentrations were higher during active disease than during remission in groups I and II, and were normal in most group III patients. Factor B and C2 values were correlated ($r=0.59; p<0.01$). Concentrations of P largely varied within the normal range. In the C2 deficent patient P was markedly increased (182% of normal) during active disease.

The serum levels of $C\bar{1}$ IA were normal or slightly increased in the patients. Other control proteins were not studied.

Whereas concentrations of factor D were increased in no group I or II patient, they were very high in group III patients, the inverted values being closely correlated with the glomerular filtration rate ($r= 0.79; p<0.01$).

C1, C2 and C3 activation. Concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA complexes were similar in all three groups, with higher values being found in samples obtained during exacerbations than during remission (fig 1). Analysis of the sera by crossed immunoelectrophoresis consistently revealed high C1s precipitates in the α_2-region. The precipitates corresponded in size to the concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA complexes, as

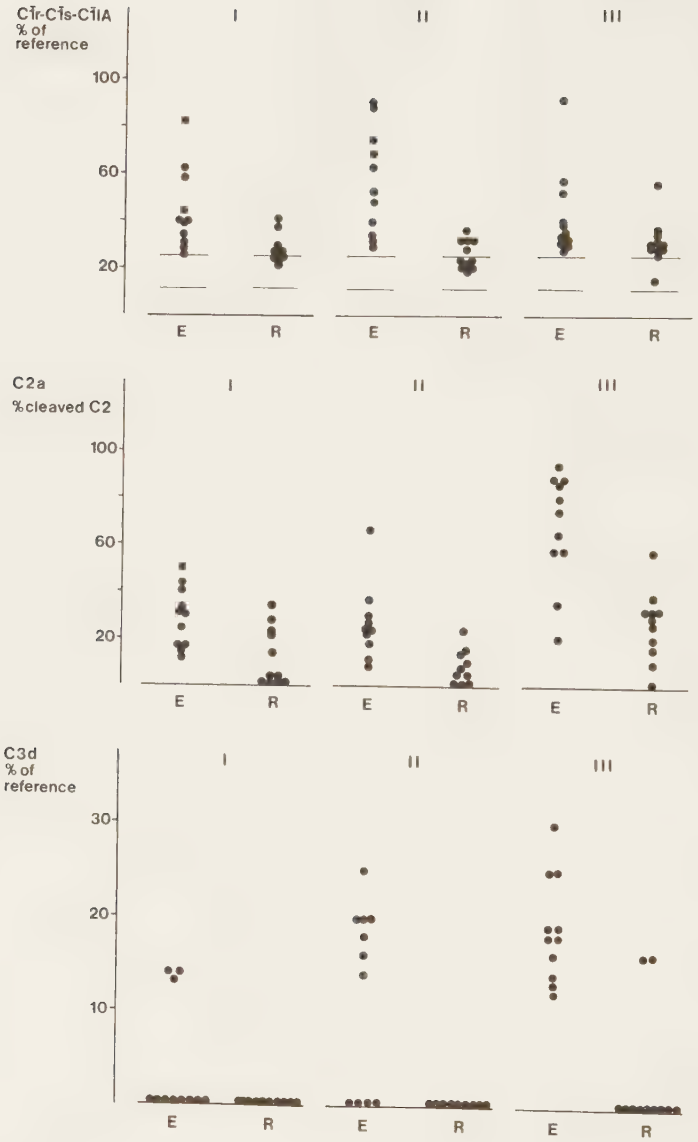


Fig 1.

Complexes of $C\bar{1}r$, $C\bar{1}s$ and $C\bar{1}$ inactivator ($C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$) in serum, and plasma C2a and C3d fragments in 33 patients with SLE. I= patients with mild SLE, II= patients with severe extra-renal manifestations and III= patients with lupus glomerulonephritis. $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ and C3d, were given in per cent of the concentrations in activated reference sera. C2a was given as the percentage of cleaved C2. The lower limit for detection of C2 cleavage was 5 - 10% and for C3d 6%. In normal sera the levels of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ ranged between 11 - 25%. C2a and C3d were not detected in plasma from 20 healthy blood donors. E: exacerbation, R: remission.

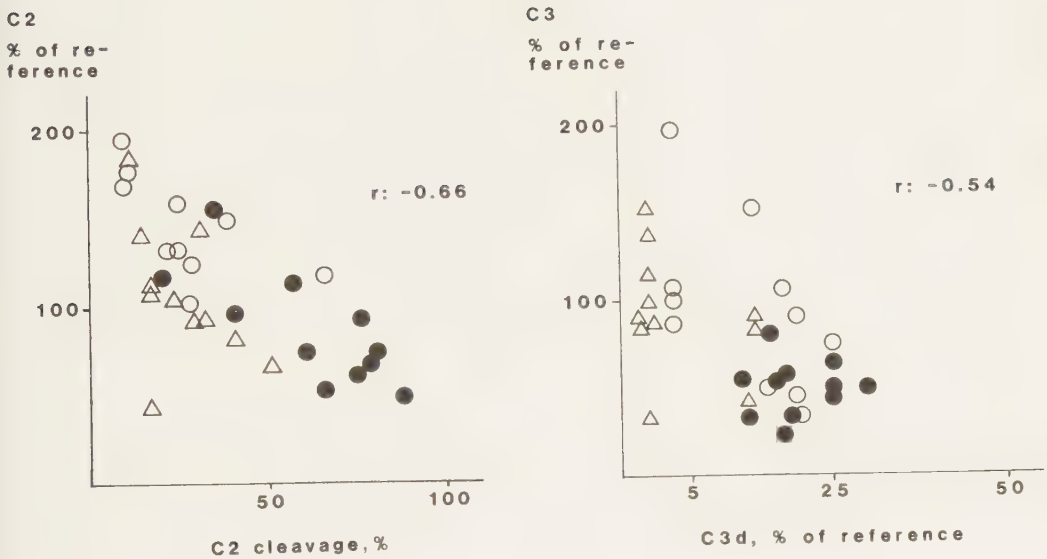


Fig 2.
Relationship between immunochemically determined C2 concentrations and C2 cleavage (left) and between C3 concentrations and C3d (right). The lower limit for detection of C2 cleavage was 5 - 10% and for C3d 6% . Group I patients are represented by triangles, group II patients by open circles and group III patients by closed circles. The patient groups were those given in Fig 1.

determined by the electroimmunoassay. Only two sera, both with very low C1q concentrations (< 5% of the normal), yielded C1s precipitates indicating the presence of zymogen C1r-C1s complexes in excess of C1q.

C2 cleavage was most pronounced in group III patients, with low C2 concentrations. An inverse relationship was found between the percentages of cleaved C2 (C2a) and the C2 concentrations (r= -0.66) (fig 2), and also between C2a and both C1q (r= -0.78) and C4 (r= -0.74).

C3d was found during exacerbation in all patients in group III, and in several group II patients (Fig 1). Three group I patients showed small amounts of C3d in plasma. No C3d was found during remission, except in two patients with renal disease in whom a silent glomerulonephritic process was probably present since they subsequently showed decreasing glomerular filtration rates. An inverse relationship was found between the concentrations of C3 and C3d (r= -0.54;p<0.01,fig 2). Low C3 (33% of normal) in absence of C3d was found in one of the group I patients, who showed persistently low C1q, C4 and C3 during the course of the study.

Taking into account the wide variation of C1 subcomponent levels, a $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$/C1q quotient was sometimes used in order to express C1 activation in relation to other factors. Such correction was perhaps justified, since C1q, C1r and C1s are present at equimolar concentrations in the native complex (11, 34). The $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$/C1q quotient was correlated with C2 cleavage (r=0.59;p<0.01). In patients with measurable amounts of C3d the quotient between C3d and C3, used as an estimate of C3d, corrected for circulating C3, correlated with the $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$/C1q quotient (r=0.63;p<0.01). C2 cleavage was also correlated with the C3d/C3 quotient (r=0.63; p<0.01).

Acute phase proteins. (Table III) Higher concentrations of CRP, α_1-antichymotrypsin, orosomocoid and α_1-antitrypsin were found in group II patients than in groups I and III. The diffe-

III

s of C-reactive protein (CRP), α_1-antichymotrypsin, α_1-antitrypsin, and orosomucoid
ra from 33 SLE patients at exacerbation and remission of the disease. Group I = 11
nts with mild SLE, group II = 11 patients with severe extra renal manifestations,
roup III = 11 patients with lupus glomerulonephritis. CRP is given in mg/l and the
acute phase reactants in per cent of the normal.

	Exacerbation		Number of patients with values higher than normal	Remission		Number of patients with values higher than normal
	median	range		median	range	
p I						
	<12	<12 - 86	5	<12	<12 - 18	1
ntichymo-rypsin	182	101 - 420	7	125	97 - 246	3
ntitrypsin	170	107 - 193	9	111	88 - 151	4
omucoid	140	92 - 356	8	136	93 - 186	8
p II						
	105	33 - 278	11	<12	<12	0
ntichymo-rypsin	250	150 - 532	11	149	96 - 202	7
ntitrypsin	186	129 - 272	11	118	74 - 175	6
omucoid	268	158 - 572	11	151	87 - 178	8
p III						
	<12	<12 - 77	2	<12	<12	0
ntichymo-rypsin	175	125 - 348	8	157	126 - 184	7
ntitrypsin	149	115 - 183	10	109	66 - 169	4
omucoid	232	140 - 344	11	147	110 - 192	9

rence areas in serum from normal adults: CRP <12 mg/l; α_1-antichymotrypsin 60 - 140 %;
ntitrypsin 62 - 117 %; orosomucoid 65 - 124 %.

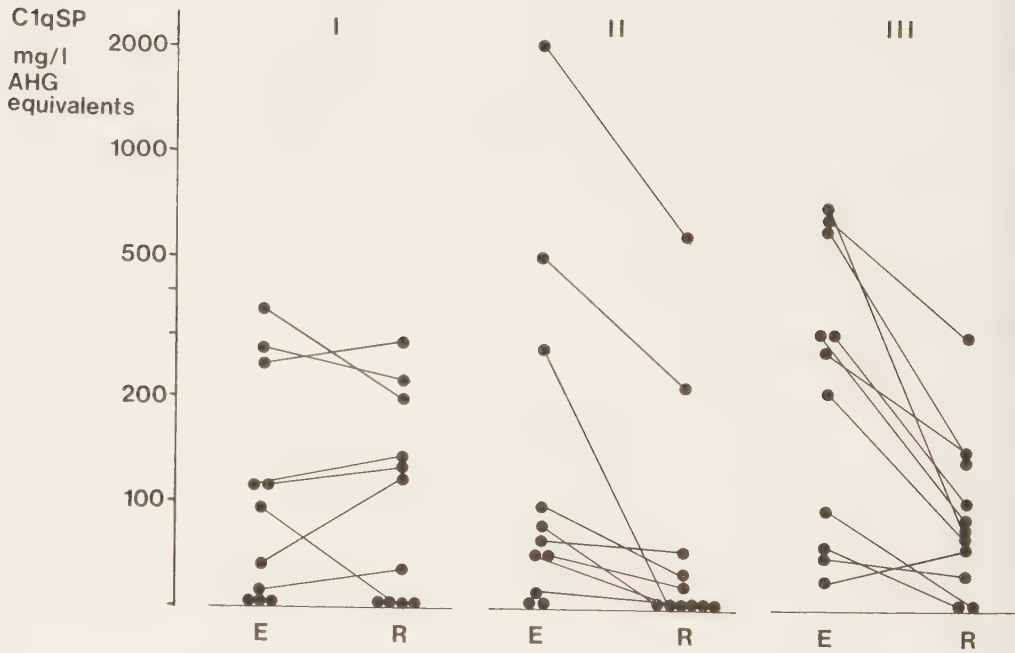


Fig 3.

Circulating immune complexes determined by solid phase C1q assay in the sera of 33 SLE patients at exacerbation (E) and at remission (R). The patient groups (I, II and III) were the same as in Fig 1. Values expressed in mg/l AHG equivalents. Normal sera yielded values <10 mg/l AHG equivalents.

rence between the groups was highly significant for CRP ($p<0.001$). However, the difference for orosomucoid between groups II and III was not statistically significant. In group II, the correlation between CRP and α_1-antichymotrypsin was marked ($r= 0.86; p< 0.01$) during active disease. In a few group III patients, the absence of measurable concentrations of CRP contrasted with increased concentrations of the other acute phase proteins. The discrepancy was striking in three patients with active glomerulonephritis, one with widespread vasculitis causing cutaneous ulcerations and two with prominent skin and joint manifestions.

Of the C components, factor B showed the most pronounced increase during active disease. The concentrations of α_1-antichymotrypsin were correlated with those of factor B ($r = 0.51; p< 0.01$). There was no significant correlation between the acute phase proteins and C1q nor with other classical pathway components.

Circulating immune complexes. (Fig 3) C1q binding immune complexes were detected in all patients with active nephritis and in most patients with extrarenal SLE. In patients with major manifestations, the levels were significantly ($p<0.01$) higher during active disease than during remission. No such difference was found in group I. Immune complex levels were correlated with C2 cleavage ($r= 0.65, p<0.01$) in active SLE. A significant correlation with the concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes was obtained if these were expressed as a $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$/C1q quotient ($r=0.52; p<0.01$). C3d showed a less marked correlation with immune complex levels ($r=0.35; p<0.05$) and C3 cleavage in patients with C3d in plasma, expressed as a C3d/C3 quotient, was not significantly correlated with immune complex levels ($r= 0.31$).

Superimposed infections. (Table IV) Among group I and II patients, several minor respiratory tract infections, two urinary tract infections (E.coli) with fever, and two Herpes Zoster infections were recorded during the study. In group III,

TABLE IV

Findings in 8 patients during periods of superimposed infection. Patient groups were the same as those in previous tables. C1q, C3, and α_1-antichymotrypsin concentrations are given in per cent of normal, $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ and C3d in per cent of the concentrations in activated reference sera, CRP in mg/l, and C1q-binding immune complexes (C1qSP) in mg/l AHG equivalents.

Patient group		Infection	C1q	C3	$C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$	C3d	Immune complexes (C1qSP)	α_1-antichymo-trypsin	CRP
I	remission	Urinary tract infection (E. coli)	104	94	28	<6	<10	96	<12
	remission	Herpes zoster	160	105	24	<6	<10	163	<12
II	remission	Herpes zoster	125	102	24	<6	<10	98	<12
	remission	Urinary tract infection (E. coli)	105	90	26	<6	<10	177	<12
III	exacerbation	Urinary tract infection (E. coli)	85	54	29	25	66	153	<12
	remission	Bronchitis	141	63	33	11	340	150	<12
	remission	Lobar pneumonia	90	76	56	13	<10	344	65
	exacerbation	Septicemia (Staph. aureus)	67	67	32	14	<10	544	<12

one urinary tract infection (E.coli), one purulent bronchitis, one lobar pneumonia and one septicemia (Staph. aureus) occurred. During infections, most patients demonstrated moderately increased or high concentrations of α_1-antichymotrypsin, orosomucoid and α_1-antitrypsin. The patient with lobar pneumonia also showed high serum concentrations of CRP (65 mg/l). In the patient, who developed septicemia during a period of active glomerulonephritis, very high concentrations of α_1-antichymotrypsin (544 %) and orosomucoid (356 %) were found, unaccompanied by any increase of CRP (<12 mg/l). Immune complexes were rarely detectable during infections.

DISCUSSION

A principal finding was that in many patients with extra renal SLE the inflammatory plasma protein response was pronounced during exacerbation, as evidenced by very high concentrations of CRP and other acute phase reactants. This was most apparent in patients with fairly severe manifestations such as fibrosing alveolitis, thrombophlebitis and serositis, conditions that might be characterized by a large inflammatory cell mass.

Since care was taken to exclude superimposed infections, and symptoms responded promptly to anti-inflammatory treatment without the addition of antibiotics, the findings argue against raised CRP as an altogether reliable indicator of bacterial infection in SLE (18). Furthermore, CRP levels were variable during superimposed infections, although the infections recorded were too few for a conclusive comparison between inflammatory plasma protein responses due to infections and to geniune SLE manifestations.

During remission, treatment with fairly large doses of corticosteroids could have contributed to findings of moderately

high concentrations of acute phase reactants other than CRP (14). Corticosteroids were clearly not responsible for the increased concentrations during flares, since the samples analyzed were obtained before treatment of the exacerbations.

CRP was usually undetectable during flares of lupus glomerulonephritis, sometimes in contrast to markedly high concentrations of other acute phase reactants. Discrepancies of this kind may have been due to to formation of complexes containing CRP (26). Evidently, the measurement of CRP alone is insufficient to assess the inflammatory plasma protein response in some patients with SLE.

Judging from concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes and of C2a, C was regularly activated during exacerbation with concomitant hypocomplementemia being fairly characteristic of patients with active glomerulonephritis (17). Obviously, the inflammatory plasma protein response had a profound effect on C component concentrations, which probably explains why hypocomplementemia was rarely seen to develop in extra-renal SLE.

The methods used did not allow strict analysis of C component levels with regard to the relative influences of synthesis and breakdown. Furthermore, the wide range of C1 subcomponent concentrations among the patients complicated the interpretation of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes as an estimate of C1 activation, although this difficulty could partly be overcome by correction of the values for the levels of C1q. An inverse relationship was found between immunochemically determined C2 and the proportion of cleaved C2. C3d, as an estimate of C3 cleavage, showed a similar relation to C3 concentrations. The findings indicated that cleavage of C2 and C3 was associated with accelerated C catabolism or possibly with decreased synthesis (5).

Factor B, as the only C component, varied in accordance with acute phase reactants such as α_1-antichymotrypsin. This is consistent with the fact that concentrations of both factor B and of C1s increase markedly in response to inflammation (23)

and with the lack of appreciable factor B consumption in SLE (29). Raised C1s values may indicate zymogen C1r-C1s complexes in excess of C1q, or high concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes (9). Assuming increased C1r and C1s synthesis in some patients, the rare occurrence of C1r-C1s complexes in the SLE sera might be ascribed to C1 activation in accord with earlier in vitro findings since it has been shown that excess C1r and C1s in C1q containing sera can be converted to $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes by classical pathway activators such as aggregated IgG (9, 11). It is not known, if the high C1q concentrations in some patients were due to increased synthesis or to C1 activation with dissociation of free C1q (9,11).

Factor D, a low molecular weight protein (15) showed raised concentrations only in patients with renal involvement, reflecting reduced glomerular filtration (30). The present findings suggest that C activation and the inflammatory plasma protein response in SLE does not affect the levels of circulating factor D.

Other factors enabling some distinction to be made between clinical groups included the concentrations of CRP, C1q, C4 and C3 and the presence of circulating C3d. Of these, C3d was perhaps the most interesting (20), since it was always found in patients with active glomerulonephritis, and was clearly a more specific indicator of C3 activation than decreased concentrations of C3.

Judging by patterns of correlation, circulating C1q binding immune complexes were at least partly responsible for classical pathway activation in the patients. Furthermore, in accordance with Abrass et al. (2) immune complexes were more abundant during exacerbations of disease activity than during periods of remission, except in patients with mild disease. $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes, C2a, and C3d also appeared to vary with disease activity, suggesting that serial measurement of these components might be of value in monitoring SLE.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Medical Research Council (B83-16X-68), the Medical Faculty, University of Lund, Konung Gustav V:s 80 års fond, Riksförbundet mot Rheumatism, Greta och Johan Kocks Stiftelser, Thorsten och Elsa Segerfalks Stiftelse, Alfred Österlunds Stiftelse, Förenade Liv, Ömsesidigt Gruppförsäkringsbolag, Stockholm, Kungliga Fysiografiska Sällskapet i Lund and Lasarettet i Lunds fonder.

REFERENCES

1. Aarden, L.A.; deGroot, E.R.; Feltkamp, T.E.W.: Chrithidia luciliae a simple substrate for the determination of anti-dsDNA with immunofluorescence technique. Ann. N.Y. Acad. Sci. 254 : 505 - 515 (1975).

2. Abrass, C.K.; Nies, K.M.; Louie, J.S.; Border, W.A. ;Glassock, R.J.: Correlation and predictive accuracy of circulating immune complexes with disease activity in patients with systemic lupus erythematosus. Arth. Rheum. 23 : 273 - 282 (1980).

3. Brandslund, I.; Siersted, H.C.; Svehag, S.-E.; Teisner, B.: Double-decker rocket immunoelectrophoresis for direct quantitation of complement C3 split products with C3d specificities in plasma. J.Immunol. Methods 44 : 63 - 71 (1981).

4. Bröchner-Mortensen, J.: A simple method for the determination of glomerular filtration rate. Scand. J. Clin. Lab. Invest. 30 : 271 - 274 (1972).

5. Charlesworth, J.A.; Williams, D.G.; Sherington, E.; Lachmann, P.J.; Peters, D.K.: Metabolic studies of the third component of complement and the glycin-rich beta glycoprotein in patients with hypocomplementemia. J. Clin. Invest. 53 : 1578 - 1587 (1974).

6. Cohen, A.S.; Reynolds, W.E.; Franklin, C.E.; Kulka, E.C.; Ropes, M.W.; Schulman, L.E.; Wallace, S.L. : Preliminary criteria for the classification of systemic lupus erthematosus. Bull. rheum. Dis. 21 : 643 - 648 (1971).

7. George, D.; Glass, D.: Quantitation of complement proteins in rheumatic disease. Clin. rheum. dis. 9 : 177 - 198 (1983).

8. Johnson, U.: Influence of ageing and polyethylene glycol on the

quantitation of C3d/dg in plasma and serum. Acta path. microbiol. Immunol. scand. Sect C. In press.

9. Laurell, A.-B.; Mårtensson, U.; Sjöholm, A.G.: Studies on C1 subcomponents in chronic urticaria and angioedema. Int. Archs. Allergy appl. Immun 54 : 434 - 442 (1977).

10. Laurell, A-B; Mårtensson, U., Sjöholm, A.: The development of simple tests for C1q, C1r, C1s, C2 and properdin; in Opferkuch, Rother Schultz: Clinical aspects of the complement system. Int. Symp., Bochum 1976, p. 12 (Thieme, Stuttgart, 1978).

11. Laurell, A.-B.; Johnson, U.; Mårtenson, U.; Sjöholm, A.G.: Formation of complexes composed of $C\bar{1}r$, $C\bar{1}s$ and $C\bar{1}$-inactivator in human serum on activation of C1. Acta. path. microbiol. Immunol. scand. Sect C 86 : 299 - 306 (1978).

12. Laurell, A.-B.; Mårtensson, U.; Sjöholm, A.G.: Quantitation of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ inactivator complexes by electroimmunoassay. Acta pathol. microbiol. Immunol. scand, Sect, C, 87 , 79-81 (1979).

13. Laurell, C.B.: Electroimmunoassay. Scand. J. Clin. Invest. Suppl. 124 : 21- 37 (1972).

14. Laurell, C.B.: Determination and interpretation of plasma proteins. Aust. Fam. Physician 5 : 36 - 41 (1976).

15. Lesavre, P.H.; Müller Eberhard, H.J. : Mechanisms of action of factor D of the alternative complement pathway. J Exp. Med. 148 : 1498 - 1509 (1978).

16. Lightfoot, R.W.; Hughes, G.R.V. : Significance of persisting serologic abnormalities in SLE. Arth. Rheum. 19 : 837 -843 (1976).

17. Lloyd, W.; Schur, P.H.: Immune complexes, complement and anti-DNA in exacerbations of systemic lupus erythematosus (SLE). Medicine 60 : 208-217 (1981).

18. Pepys, M.B.: Serum C-reactive protein, serum amyloid P-component and serum amyloid A protein in autoimmune disease ; in Holborow, Clinics in immunology and allergy, vol 1 pp 77 - 101 (1981).

19. Pepys, M.B.: C-reactive protein : the role of an ancient protein in modern rheumatology. Clin.Exp. Rheumatology 1 : 3 - 7 (1983).

20. Perrin, L.H.; Lambert, P.H.; Miescher, P.A.: Complement breakdown products in plasma from patients with systemic lupus erythematosus and patients with membranoproliferative or other glomerulonephritis. J. Clin. Invest. 56 : 165 - 176 (1975).

21. Rynes, I.R.: Inherited complement deficiency states and SLE. Clin. Rheum. Dis. 8 : 29 - 47 (1982).

22. Schur, P.: Complement and lupus erythematosus. Arth. rheum. 25 : 793 -798 (1982).

23. Schutte, M.; DiCamelli, R.; Murphy, P.; Sadove, M.; Gewurz, H.: Effects of anestesia, surgery and inflammation upon host defence mechanisms. I. Effects upon the complement system. Int. Archs. Allergy appl. Immun. 48 : 706 - 720 (1975).

24. Scullion, M.; Balint, G.; Whaley, K.: Evaluation of the C1q solid-phase binding assay for immune complexes. A clinical and laboratory study. J. clin. lab. Immunol. 2 : 15-22 (1979).

25. Siegal, S.: Nonparametric statistics for the behavioral sciences (McGraw Hill, New York 1956).

26. Siegel, J.; Rent, R.; Gewurz, H.: Interactions of C-reactive protein with the complement system. I. Protamine-induced consumption of complement in acute phase sera. J.Exp. Med. 140 : 631 - 647 (1974).

27. Sjöholm, A.G.: Complement components in normal serum and plasma quantitated by electroimmunoassay. Scand. J. Immunol. 2 : 25 - 30 (1975).

28. Sjöholm, A.G.; Sturfelt, G.: Cleavage of C2 in pathological serum and plasma studied by crossed immunoelectrophoresis. Acta. path. microbiol. Immunol. scand. Sect C Immunol. In press.

29. Sturfelt, G.; Sjöholm, A.; Svensson, B.: Complement components, C1 activation and disease activity in SLE. Int. Archs Allergy appl. Immun. 70 : 12 - 18 (1983).

30. Sturfelt, G.; Truedsson, L.; Thysell, H.; Björk, L.: Serum level of complement factor D in systemic lupus erythematosus (SLE) - an indicator of gloerular filtration rate. Acta. Med. Scand. In press.

31. Tan, E.M.; Kunkel, H.G. : Characteristics of a soluble nuclear antigen precipitating with sera of patients with systemic lupus erythematosus. J.Immunol. 96 : 464 - 471 (1966).

32. Tan, E.M.; Cohen, A.S.; Fries, J.F.; Masi, A.T.; McShane, D.J.; Rothfield, N.F.; Schaller, J.G.; Talal, N.; Winchester, R.J. : The 1982 revised criteria for the classification of systemic lupus erythematosus. Arth. Rheum. 25 : 1271 - 1277 (1982).

33. Truedsson, L.; Sturfelt, G.: Human factor D of the alternative pathway: Purification and quantitation by enzyme amplified electroimmunoassay. J. Immunol. Methods. 63 : 207 - 214 (1983).

34. Ziccardi, R.J.; Cooper, N.R.: The subunit composition and sedimentation properties of human C1. J. Immunol. 118 : 2047 - 2052 (1977).

SEQUENTIAL STUDIES OF COMPLEMENT ACTIVATION IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Running headline: C activation in SLE.

Gunnar Sturfelt[1], Ulf Johnson[2] and Anders G. Sjöholm[2]

From the Departments of Rheumatology[1] and Immunology[2], Institute of Microbiology, University Hospital, Lund, Sweden

Correspondance: G.Sturfelt, Department of Rheumatology, University Hospital, S - 221 85 Lund, Sweden.

ABSTRACT

C1 and C3 activation, measured as $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ inactivator ($C\bar{1}s$-$C\bar{1}r$-$C\bar{1}IA$) complexes in serum and circulating C3d were studied in serial samples from 33 patients with SLE. All patients demonstrated exacerbations during observation periods of 10-30 months and were divided into groups according to principal clinical features (mild SLE, severe extra-renal SLE and lupus glomerulonephritis). Increased C1 activation was consistently found during exacerbation. C3d in plasma was a feature associated with flares of severe disease. Activation of C1, but not of C3, was documented before flares of disease activity, but such predictive information was mostly restricted to patients with extra-renal disease. C2 cleavage in plasma, studied serially in a few patients, appeared to be closely associated with C1 activation. Circulating immune complexes, measured with solid phase C1q assay, did not always increase before development of clinical manifestations. Remission of symptoms was parallelled by decreasing concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ and of, when present, C3d. Similar findings were made for immune complexes but only in severe disease. Persisting C3d was observed in three patients, who subsequently developed renal failure. C1q levels were transiently low during flares of lupus glomerulonephritis, but otherwise the concentrations of C1q, C4 and C3 did not show consistent patterns of variation in relation to disease activity.

Key words : Complement components, complement activation, systemic lupus erythematosus.

IV

INTRODUCTION

Opinion differs as to the efficacy of iterative measurement of complement (C) components in monitoring SLE disease activity (5, 23). Tests for antibodies to native DNA (nDNA) and C1q-binding immune complexes have been reported to provide predictive information in SLE (2,31). A disadvantage with assays for anti-nDNA, and perhaps with those for C1q binding immune complexes, is that not all patients with SLE demonstrate positive tests during active disease.

In active SLE, C1 activation is often pronounced (28) and evidence of C3 activation has also been reported (19,21). In this study we have tried to analyze the significance of C1 activation, as it was reflected by presence in increased amounts of complexes containing $C\bar{1}r$, $C\bar{1}s$ and $C\bar{1}$ inactivator (13), and of C3 breakdown, by measuring C3d, in relation to changes in disease activity in SLE patients. C2 cleavage was studied during flares of disease activity, and in a few patients also in serial samples. Three patient groups, representing mild SLE, severe extra-renal SLE and lupus glomerulonephritis were followed for 10 - 30 months. The results of the C activation assays were compared with the concentrations of C1q, C4 and C3, and with C1q binding immune complexes measured by a solid phase assay. In addition, C2, factor B and properdin levels were determined in most of the samples. In a another study of the same patients, complement components and complement activation were investigated in relation to the inflammatory plasma protein response (30).

MATERIAL AND METHODS

Patients. A standardized SLE control program was started at the Department of Rheumatology during 1980. Clinical and laboratory investigations were carried out, and serum and EDTA plasma samples collected at regular intervals of two months (6 - 8 weeks), or as appropriate to the course of the disease.

Patients presenting variations of disease activity with one or more exacerbations during an observation period of 10 - 30 months were included in the study consecutively. The presence, during the observation period, of minor or major manifestations as defined by Lightfoot and Hughes (16), constituted the basis for assignment of the patients to three clinical groups : patients with minor manifestations only (group I), patients with severe (major) extra-renal manifestations (group II), and patients with active renal disease (group III). Group I consisted of 11 women, and groups II and III of 3 men and 8 women each. On inclusion in the study, the ages of the patients were between 16 and 60 years (median 38) in groups I and II, and 32 years in group III. The duration of the disease from its diagnosis was 0 - 22 years, median 2 years in each group. Four or more of the criteria proposed by the American Rheumatism Association were fulfilled by the 33 patients (6,33). All patients were anti nuclear antibody (ANA) positive at least on one occasion. In immunodiffusion tests (32) anti-Sm antibodies were detected in two patients in each group, anti-ribonucleoprotein (RNP) antibodies in two patients in group I and in three in each of groups II and III. During active disease anti-nDNA (1) antibodies were detected in 4 patients in group I, 7 patients in group II and 9 in group III.

Evaluation of clinical manifestations. At the time of each clinical attendance it was decided if changes in disease activity were present. The evaluation was made without access to results of immunological tests.

Renal disease was considered active where one or more of the following observations were made: 1. Debut of hematuria or pathological casts; 2. Persistant pathological urinary sediment in conjunction with impaired renal function (S-creatinine, CrEDTA clearance); 3. Onset of proteinuria >0.5 g/24 h. Renal disease was rated as inactive where renal function (S-creatinine, CrEDTA clearance) improved or did not deteriorate over an observation period of more than six months (i.e. three clinical attendances) and where urine sediment or blood pressure remained unchanged.

Clinical evaluation and medical treatment of patients with renal involvement was arranged in consultation with Dr. H. Thysell, Department of Nephrology, University Hospital, Lund.

All clinical evaluations of neuropsychiatric manifestations were made by trained physicians at the Neurological and Psychiatric Departments, University Hospital, Lund. Laboratory investigations included computerized tomography, visual evoked response EEG, blood flow measurement (Xe_{133}) and routine liquor analysis.

Active serositis (pleuritis, pericarditis) was diagnosed by pain accompanied by a rub or an effusion, abnormal chest X-ray, or ECG changes consistent with pericarditis. All exudates were examined with cultures, glucose analysis, and leukocyte counts .

Differential diagnosis against infection. In patients with fever or major manifestations, investigation included cultures from blood, urine and nasopharynx, and serological screening for

viral infection. Further investigations were performed as appropriate to the clinical picture. Periods with proven infection were excluded from the present study.

Sampling of serum and plasma. After venesection clotting was allowed to procede for 1h at +20°C and thereafter for 1h at +4° C. Blood collected in EDTA at 5 mmol/l was handled in the same way. After centrifugation serum and plasma were frozen in aliquots and stored at -80°C until analyzed.

Complement components. C1q, C4, C2, C3, B and P were measured as previously described (14, 27), values being given in per cent of the concentrations in a pooled reference serum. Normal reference areas were : C1q: 78 - 130%; C4: 53 - 207%; C2: 75 - 163%; C3: 70 - 136%; B: 59 - 154%; P: 57 - 153%.

To measure C1 activation complexes of $C\bar{1}r$, $C\bar{1}s$ and $C\bar{1}$ inactivator ($C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA) were quantitated by electroimmunoassay (15), values being given in per cent of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA in a normal serum pool after activation of C1 with heat aggregated human IgG (AHG) (15). In normal sera the concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA ranged between 11 and 25 % of that in the reference.

C2 cleavage (C2a) was assessed by crossed immunoelectrophoresis (29), the proportion of cleaved C2 (C2a) in EDTA plasma being expressed as a percentage of the area covered by precipitated C2 protein. Depending on the C2 content in the samples, cleavage of 5 - 10% of C2 present in the samples could be detected. No C2a was found in EDTA plasma obtained from healthy donors (n= 20).

C3d, defined as molecules carrying C3d but not C3c antigen, was measured in plasma by a "double-decker" electroimmunoassay with anti C3c and anti C3d (4) as modified by Johnson (12). Polyethylene glycol was not used in preparation of the samples before analysis. C3d in plasma was given as a percentage of the C3d in a reference pool, treated with inulin (100 mg/ml, 37°C 120 min). No C3d was detected in plasma from 20 healthy individuals. This was in contrast to the findings of Brandslund

(4). However, we used half the amount of plasma for analysis, which might have contributed to the apparently lower sensitivity of our assay. The lower limit for detection of C3d was about 6% of the C3d concentration in the reference preparation.

Circulating immune complexes. Circulating immune complexes were measured by solid phase C1q binding assay (C1qSP) according to Scullion et al. (25) with slight modifications (28). The values were given as AHG equivalents in mg/l. Control sera yielded values < 10 mg/l.

Statistical evaluation. For the analysis of laboratory data, samples obtained at the first indication of increasing clinical disease activity, and approximately 2 and 4 months earlier were used. The time interval between the samples obtained at first indication of active disease and at the closest preceding control varied between 4 and 8 weeks (median 6 weeks). Correlation between clinical improvement and normalization of the laboratory variables was also studied analyzing samples taken at maximal disease activity and at two and four months later, when the patients were considered to be in remission.

For statistical evaluation, only one flare in each patient was used. Where more than one exacerbation were recorded in a given patient, the first was chosen.

Differences between paired values were evaluated by the Wilcoxon matched pairs signed ranks test (26). Differences between independent values was analysed by the Mann-Whitney U-test (26).

RESULTS

Clinical manifestations. A survey of the clinical features at the exacerbations recorded during the study is given in Table I.

Group I. Cutaneous and musculo-articular symptoms were most prominent in these patients, several of whom had fever during flares. In an anti-nDNA negative patient glomerulonephritis had previously been verified by renal biopsy. The patient had, however, normal glomerular filtration rate and no signs of active renal disease during the study.

Group II. Pleuropericarditis was found in 8 patients, two of whom also had neuropsychiatric manifestations: a 52 year old woman having hemiplegia and a 40 years old man an organic brain syndrome. Defect fibrinolysis was found in a 25 year old woman, who was in a poor general condition with fever, femoral thrombosis and pulmonary embolism, when included in the study. A 68 year old woman had active fibrosing alveolitis. During the study, a patient with homozygous C2 deficiency developed a flare with acute pancreatitis, fever without evidence of infection, arthritis and exanthema. One anti-nDNA positive patient had occasional microscopic hematuria, but further investigations failed to provide evidence of glomerulonephritis.

Group III. 11 patients with evidence of active glomerulonephritis were studied, in 10 of whom renal disease had been confirmed by renal biopsy. Five of the patients with active glomerulonephritis exhibited other major manifestations as well. Two men, 17 and 24 years old, had neurological symptoms with hemiplegia and hemianopsia respectively, the latter also having cardiomyopathy and at the end of the study, gastrointestinal involvement with possible pancreatitis. A 53 year old woman developed pulmonary embolism during a period of active

Table I

Clinical manifestations during flares in 33 SLE patients. None of the patients showed more than 2 flares during the observation period. Group I: patients with only minor manifestations, group II: patients with major manifestations but no evidence of active renal disease, group III: patients with active lupus glomerulonephritis.

Group I (11 patients)	No of flares
Arthritis	3
Arthritis + exanthema and/or Raynauds phenomeneon	12
Arthritis + myositis	1
Group II (11 patients)	
Pleuropericarditis + miscellaneous minor symptoms	7
Pleuropericarditis + cerebral vasculitis (hemiparesis) + arthritis	1
CNS-involvement + pleuropericarditis	1
Pancreatitis + fever + arthritis	1
Pulmonary embolism + arthritis + exanthema	1
Fibrosing alveolitis + arthritis	1
Cutaneous vasculitis + cutaneous ulcerations	1
Group III (11 patients)	
Glomerulonephritis + miscellaneous minor symptoms	10
Glomerulonephritis + pulmonary embolism + exanthema	1
Glomerulonephritis + CNS-involvement + arthritis	1
Glomerulonephritis + CNS-involvement + cardimyopathy	1
Glomerulonephritis + cutaneous vasculitis with ulcers	1
Glomerulonephritis + Thrombocytopenia	1

nephritis. One patient had widespread cutaneous vasculitis with ulcers, and another patient thrombocytopenia.

Leukocytopenia, that could not be explained by medical treatment, was observed at flares in 12 patients, four in each of the three groups.

By definition, group I patients could be controlled by antimalarial drugs or Prednisolone in dosages < 20 mg/day, or a combination of both. Patients in groups II and III required dosages of Prednisolone > 20 mg/ day, and were always hospitalized during flares. In addition to corticosteroids, Azathioprine was used to treat three patients in group II and nine in group III at one time or another during the study. One patient was treated with cyclophosphamide during a part of the observation time. In six of the patients with renal involvement, plasma exchange was performed for short periods (1 - 2 weeks) as an adjunct to immunosuppression. One patient was also treated with extra-corporal plasma absorbtion to immobilized protein A during a short period of time. Serum and plasma samples obtained within one week after plasma exchange were excluded from the investigation.

Complement component concentrations during flare. Concentrations of C1q, C4 and C3 were temporarily reduced in 8 of 11 patients during active nephritis. In group II, C1q was low in five patients and high in four, C4 was low in two patients and high in none, and C3 was low in four patients and high in two. Simultaneously low C1q, C4 and C3 were seen in two patients in group II, one in group I. In group I, one patient with active disease had low C1q, four had low C4, and two had low C3 while five patients had increased C1q, none increased C4 and two had high C3. One patient in group II had a homozygous C2 deficiency. Low C2 concentrations were found in two group I and six group III patients but in none of the group II patients. High C2 levels was present only in three group II patients. During active disease, factor B was high or normal and P concentrations varied within normal range in all cases with the exception of

Table II

$C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ inactivator ($C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA) complexes and C1q binding immune complexes measured in serum and C2 cleavage (C2a) and C3d fragments in plasma from 33 SLE patients. Group I = patients with mild SLE, group II = patients with severe extra-renal manifestations, group III = with active lupus glomerulonephritis. Number of flares with normal values are given within brackets.

	Group I (16 flares)			Group II (13 flares)			Group III (15 flares)		
	range	median		range	median		range	median	
$C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA (per cent of reference)	22-82	41	(2)	29-90	48	(1)	27-91	36	(0)
C2a (per cent cleaved C2)	<5-50	23	(1)	8-66	24	(0)	31-94	71	(0)
C3d (per cent of reference)	<6-14	<6	(13)	<6-25	15	(5)	12-30	18	(0)
C1qSP (mg/l AHG equivalents)	<10-350	90	(3)	<10-2000	74	(2)	20-690	205	(0)

Normal samples: $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA: 11-25 %; C2a:5-10 %: C3d: < 6 %; C1qSP: < 10 mg/l AHG equivalents.

the patient with homozygous C2 deficiency, in whom P was markedly high during her major flare.

Activation of C1, C2 and C3. During flare, the patients had increased concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA complexes or of C2a fragments or both (Table II). Two group I patients showed normal $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA values during one of their flares, and in another group I patient C2 fragments were not found. Increased concentrations of C3d were recorded in 3 out of 16 minor flares in group I patients (Table II). For technical reasons, C2a was expressed relative to the concentrations of C2 antigen, which implied that the values were not strictly comparable with those of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA complexes and C3d (Table II). However, the estimates of C1, C2 and C3 activation were fairly closely correlated, provided that $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA complexes and C3d were corrected for the C1q and C3 concentration, respectively (30). C3d concentrations were consistently increased during active glomerulonephritis and moderately increased in 8 out of 13 flares in patients with major extra-renal manifestations (Table II). Circulating C3d was rarely found in quiescent disease.

Immune complexes. Circulating C1q binding immune complexes were detectable during most of the flares studied (Table II). Two patients, one with skin and joint manifestations and one with serositis, did not show immune complexes at any time. However, raised $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA were found, together with C2a and C3d.

Sequential studies. Ten group I, six group II and eight group III patients could be studied during a pre-flare period of at least four months. During the four months preceding first clinical evidence of exacerbation, serum concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA increased in most patients (Fig 1). Changes of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA concentrations from four to two months prior to flare were statistically significant in group I and when patients in the three groups were studied as a whole (Fig 1).

Increasing amounts of circulating C3d were detected during incipient flare in all patients with renal involvement, but

Fig 1.
Changes in concentrations of C1r-C1s-C1 inactivator complexes (C1r-C1s-C1IA), C3d fragments, C1q-binding immune complexes (C1qSP), C1q and C4 during intervals preceding the first signs of clinical flare (F) in patients with mild SLE (group I), severe extra-renal SLE (group II) and with lupus glomerulonephritis (group III). The interval between four and $\leq$ two months (4 - 2) before and the interval between $\leq$ two months before flare and the development of symptoms were studied. Ten patients in group I, six in group II and eight in group III were observed during pre-flare periods. For C1qSP and C3d, patients with detectable amounts of C1q binding immune complexes and C3d fragments are presented. C1r-C1s-C1IA and C3d were expressed as percentages of the concentrations in activated reference sera, C1qSP as AHG equivalents (mg/l) and C1q and C4 as percentages of the concentrations in pooled reference serum. Changes of laboratory values were given as the numerical difference between values obtained at the start and at the end of each interval. The patient with homozygous C2 deficiency is indicated by open circles.

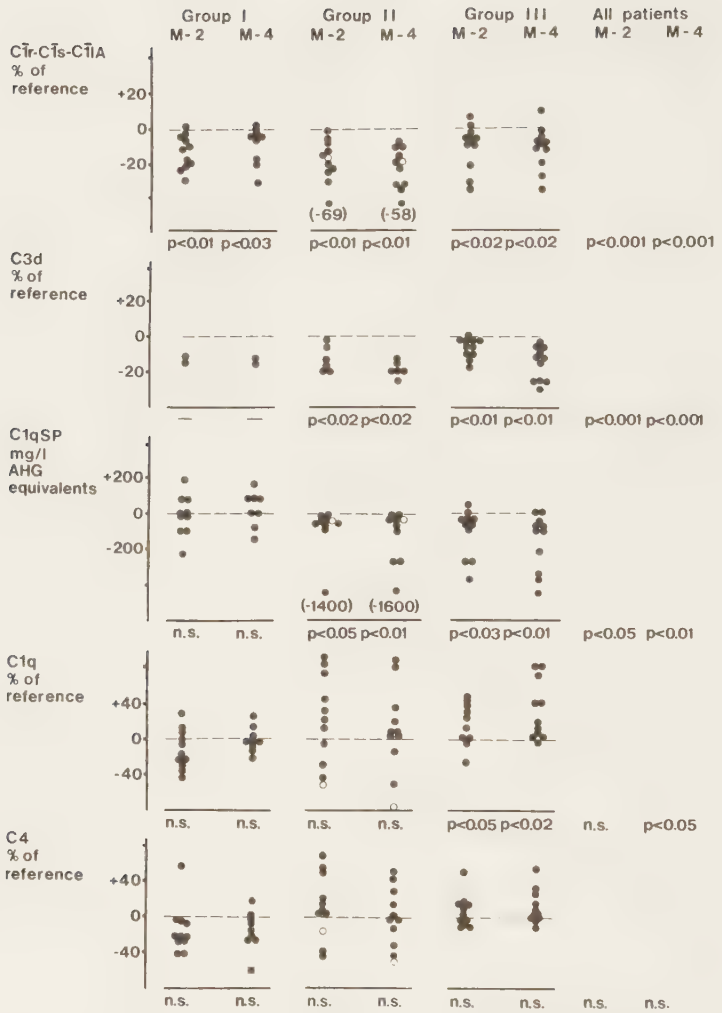


Fig 2.
Changes in concentrations of C1r-C1s-C1 inactivator complexes (C1r-C1s-C1IA), C3d fragments, C1q binding immune complexes (C1qSP), C1q and C4 during treatment of flares. The intervals were those between start of treatment at the time of maximal disease activity (M), and after two months (M - 2) and four months (M - 4) of treatment. Patient groups were the same, and numerical differences between values given as in Fig 1. For C1qSP and C3d, patients with detectable amounts of C1q binding immune complexes and C3d fragments are presented. In two group I patients samples were not obtained at four months after M.

examination of the C3d concentrations at two and four months before flare provided no predictive information. C2a was measured sequentially in two patients from each clinical group and paralleled disease activity with a sensitivity that appeared to be similar to that of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA.

The serum concentrations of C1q, C4 and C3 and immune complexes as measured by C1qSP did not permit prediction of exacerbations in the patients studied, except that, in those with renal involvement, the serum concentration of C1q decreased significantly between two months before flare and the first indications of increasing disease activity.

During remission, decreasing concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$IA, C3d and of C1q binding immune complexes paralleled clinical improvement in patients with major manifestations (Fig 2). In patients with renal involvement, clinical improvement was also paralleled by increasing C1q concentrations. In group III patients C3 concentrations increased almost significantly ($p=0.05$) during the 4 months interval following maximal disease activity. Other changes of C3 and of C4 during the intervals studied were clearly at random. C3d persisted in three patients, even though clinical findings indicated stabilization of the disease for several months; within a year, however, these patients showed deteriorating renal function. Persistence of circulating C3d was not consistently associated with low C3 values. In patients without renal involvement, no C3d was detected during inactive disease.

Several patients demonstrated persistently low complement levels throughout the study. Two patients, one with glomerulonephritis and another with a fever episode and active skin and joint symptoms, had markedly low C1q, C4 and C3 concentrations even when the disease was unmistakably quiescent. Three patients without renal involvement had persistently low C4 concentrations, and one group I patient had low C4 and C3 concentrations in serum both in active and inactive disease. In the patient with homozygous C2 deficiency increased

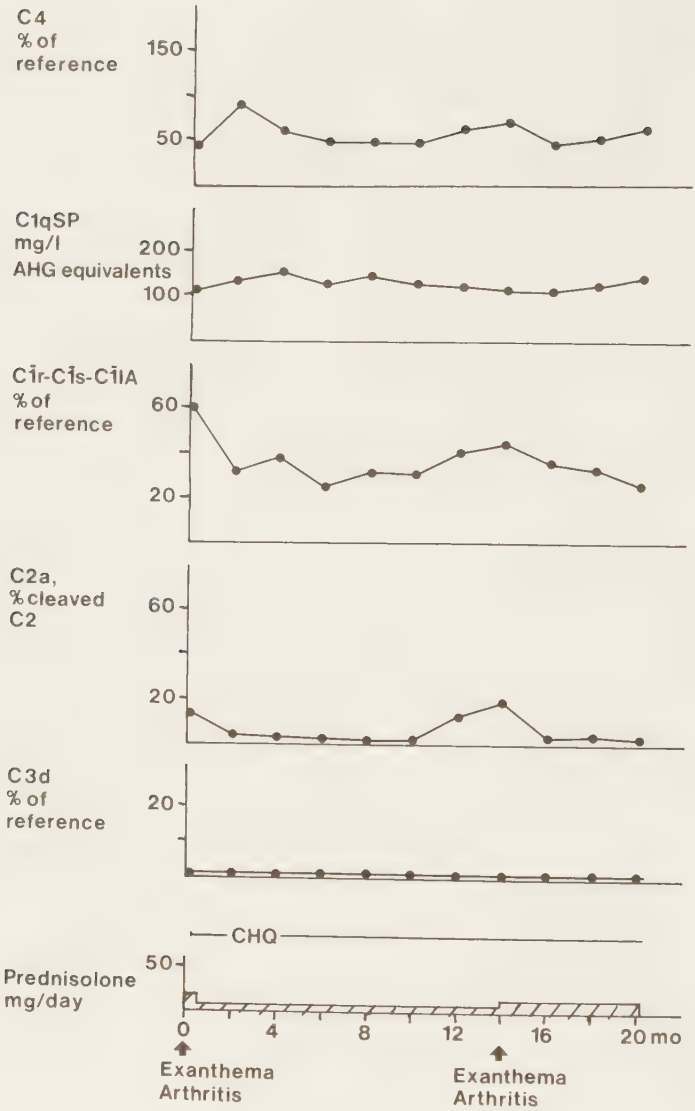


Fig 3.

Arthritis and cutaneous manifestations in a 29 year old woman. No C3d was detected, while C1̄r-C1̄s-C1̄IA and C2a were found in increased amounts before and during active disease. mo=months; CHQ=chloroquine. C1̄r-C1̄s-C1̄IA and C3d were expressed as % of the concentrations in activated reference sera, C2a as % of cleaved C2, C4 as % of the concentrations in pooled reference serum and C1qSP as AHG equivalents (mg/l).

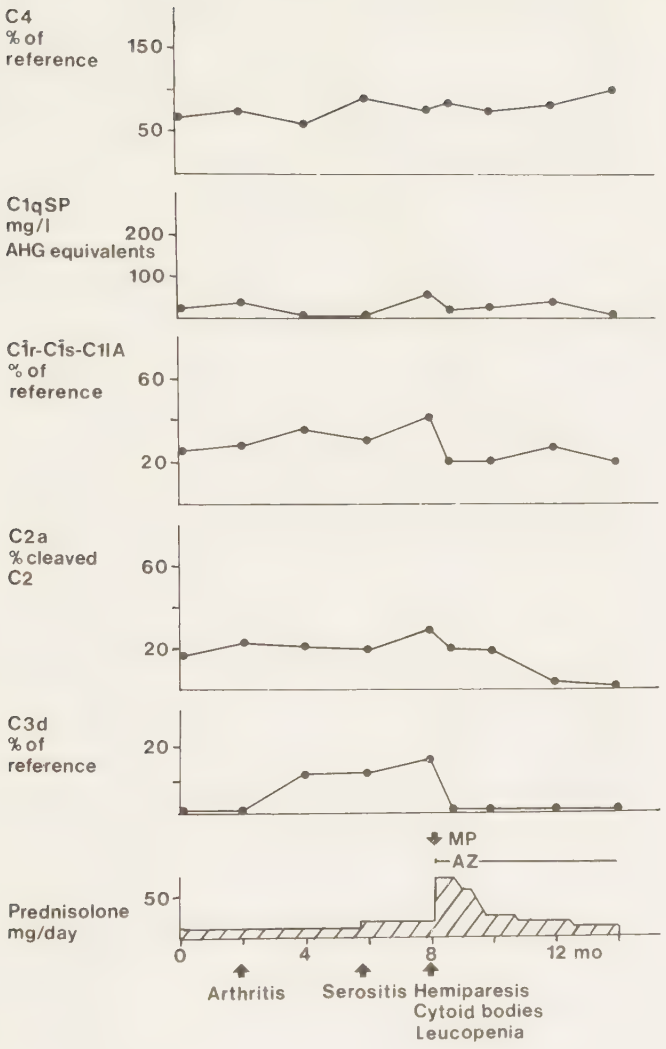


Fig 4.

Severe extra-renal manifestations including fever, serositis, neurological symptoms (hemiplegia) in a 52 year old female. During exacerbation C3d and C2a were found in plasma together with increased serum concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA. After the institution of pulse dosages of cortocosteroids circulating C3d was not detected. AZ=azathioprine; MP=methylprednisolone. Values are given as in fig 3.

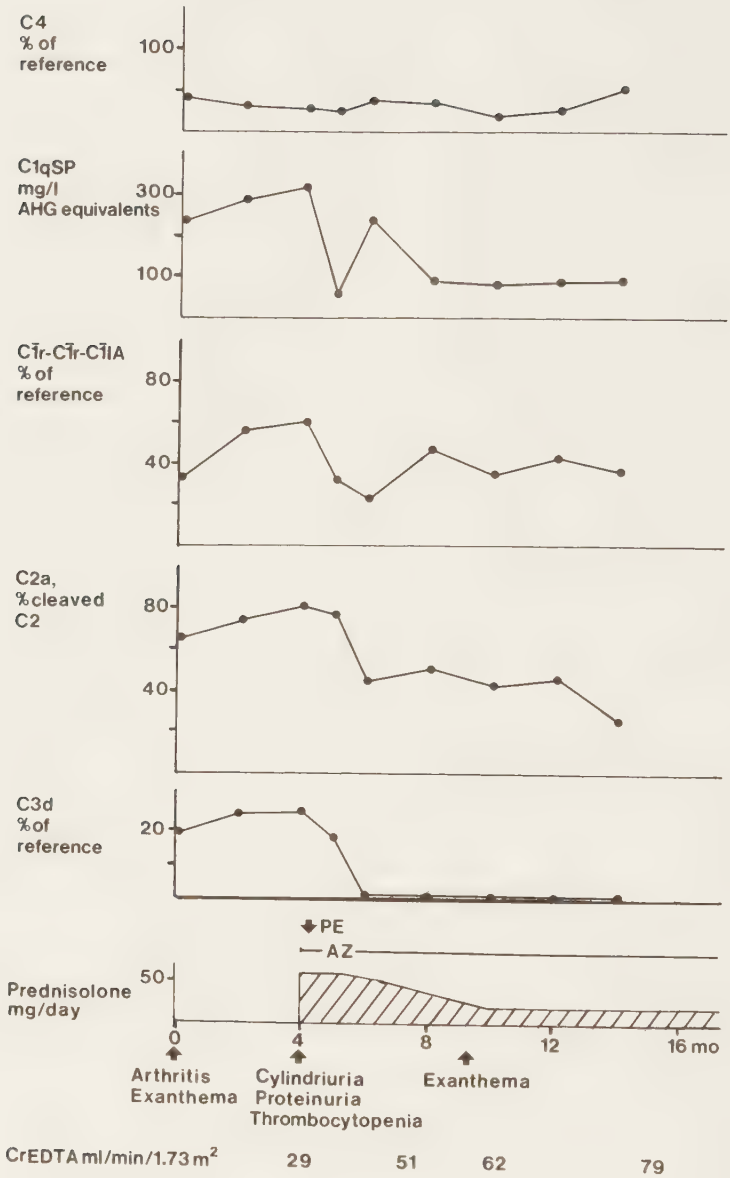


Fig 5.

Serial data on a 20 year old woman, who entered the study a few weeks after onset of arthritis, exanthema and slight proteinuria (<0.5 g/day). More severe renal involvement developed during the following months. C3d dissappeared, while C1r-C1s-C1IA persisted at somewhat lower concentrations after improvement of renal function, the patient then having minor symptoms from skin and joints. PE= plasma exchange; AZ=azathioprine. Values are given as in fig 3.

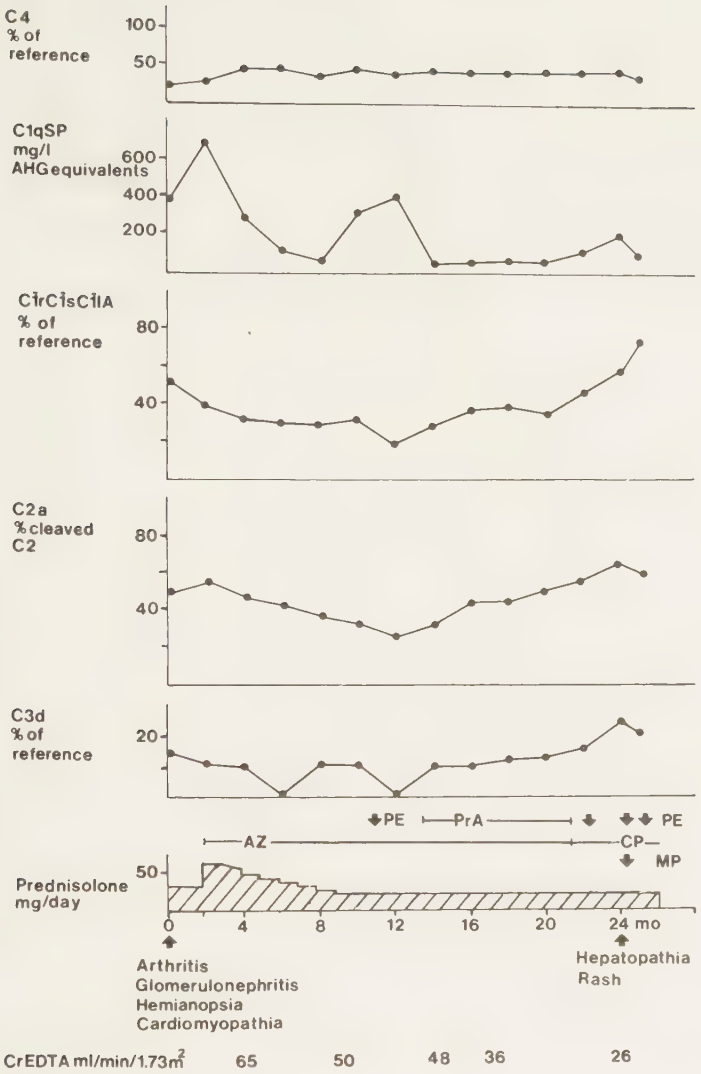


Fig 6.

Serial data on a 22 year old man who entered the study with evidence of active glomerulonephritis, cardiomyopathia and CNS-manifestations (hemianopsia). A stabilized clinical picture was achieved after institution of immunosuppresive treatment. Because of gradually decreasing renal function plasma exchange and plasma treatment with immobilized protein A were tried out, but without success. Toward the end of the observation period, increased disease activity was seen with fever, cutaneous and gastro-intestinal manifestations. The clinical course was reflected by evidence of C1 and C2 activation and by the persistence of circulating C3d. CP= cyclophosphamide; PE= plasma exchange; PrA= Protein A treatment; AZ= azathioprine; MP= methylprednisolone. Values are given as in fig 3.

concentrations of C1q, C4, C3 and P were found during severe flare, while factor B remained normal. Persistently low C2 concentrations were found in two group I patients and three group III patients. One of the two group I patients, with C2 values ranging between 38% and 43% of normal, showed less C2 cleavage during active disease than other patients with similar C2 levels.

The findings in four representative patients are summarized in the figures 3 - 6.

DISCUSSION

The investigation was designed so as to permit evaluation of C activation in relation to trends of disease activity in different clinical categories of SLE - i.e., patients with mild disease, and those with severe disease with or without renal involvement. The study was restricted to patients with at least one flare during the observation period.

Concentrations of C components in the circulation provide less information with regard to C activation than does the presence of C component complexes and fragments generated in the course of C activation (7). In accordance, hypocomplementemia was not a consistent finding during flares while increased amounts of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes and C2a fragments could be demonstrated in the patients. Furthermore, C3d fragments were usually present in patients with severe disease even when concentrations of C components were not decreased.

Although EDTA prevents recruitment of classical and alternative pathway C3 convertases, hydrolysis of C3 occurs spontaneously with generation of C3b-like molecules that can be further degraded also in EDTA plasma (20). This, together with the possibility that small amounts of C3d are present in the

circulation under physiological conditions is consistent with previous findings of C3d in normal EDTA plasma (3,21). The sensitivity of the C3d assay used in the present study was adjusted to avoid influence of such background effects. The omission of polyethyleneglycol pretreatment of samples might add to the reliability of the assay and furthermore allows detection both of low molecular weight C3d and C3d containing complexes (11).

In at least some patients with normal or high concentrations of C components, the breakdown of C components was probably balanced by their enhanced synthesis due to the inflammatory plasma protein response (24). This was particularly true of patients with some extra-renal manifestations, who, during flares, showed markedly high concentrations of several acute phase reactants including C-reactive protein (30).

Decreasing C4 concentrations (8), and raised concentrations of C1q binding immune complexes (2) and of nDNA antibodies (31) have been reported to precede the onset of clinically evident flares in SLE patients. In the present study, increased C1 activation was found to precede exacerbations by several weeks, at least in patients with extra renal manifestations. Analysis of preflare C activation in patients with glomerulonephritis was compromised by the occasional presence of minor extra-renal symptoms during periods of low active renal disease, and perhaps by rapid development of exacerbations in lupus glomerulonephritis (17).

In general, clinical improvement following treatment of SLE exacerbations was closely paralleled by decreasing concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes and, when present, of C3d. Circulating C3d appeared to be particularly significant in lupus glomerulonephritis, since such fragments were invariably found from the onset of exacerbations in patients with renal involvement, and persisted only in those who subsequently developed progressive renal failure.

Perrin et al. (21) previously observed elevated C3d levels in SLE, the highest values being associated with severe disease and glomerulonephritis. In possible conflict with these and the present findings Morrow et al. (19) reported that C3d levels bear little relationship to disease activity in SLE. The design of their study, according to which patients were not classified with regard to principal clinical features, such as glomerulonephritis, could be a reason for the different conclusions.

Judging from our studies of C2 cleavage in serial samples from a few patients, activation of C2 in SLE was more closly associated with activation of C1 than of C3.

Circulating immune complexes measured by C1qSP were also found to vary with disease activity, except in patients with mild disease. This was in partial agreement with the findings of Abrass et al.(2), although we were unable to confirm that the assay predicts the development of flares. Some patients demonstrated transiently decreased immune complex concentrations during the early phase of exacerbations that could not be explained by institution of immunosuppressive or corticosteroid treatment. A similar decrease of circulating anti-nDNA antibodies related to development of major SLE manifestations was reported by Swaak et al. (31).

The precise mechanisms by which treatment reduced the concentrations of C activation products are not known, but the findings suggest that the apparent decrease of C activation could be related to the influence of treatment on C-activating immune complexes. Difficulties associated with interpretations of this kind have been discussed by Petz et al. (22). Concerning the patients treated with plasma exchange it should be pointed out that no samples obtained within a week of such treatment were used in the present investigation.

With the exception for C1q, found to be transiently low during flares of lupus nephritis, none of the C components showed

consistent patterns of variation in relation to trends of disease activity. Interestingly, some patients showed persistently low concentrations mainly of C4 and C2, apparently unrelated to variations in disease activity or to C activation. Genetically determined, partial defects of C components might have been present, since such aberrations have been reported to be common in SLE (9).

In one patient with severe extra-renal SLE, known to have a homozygous C2 deficiency, C1 activation was found during active disease, but no evidence of alternative pathway activation, which was in contrast to previous findings in a C2 deficient patient with renal involvement (10).

To conclude, it was suggested that sequential determinations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}IA$ complexes and of C3d are useful indicators of disease activity in SLE and provide more reliable information than do measurements of C1q binding immune complexes or of C component levels. C1 activation was a fairly consistent feature in flares of disease activity, while C3d in plasma appeared to be a marker of severe organ involvement. The results should encourage further investigation of C activation assays in SLE, including the detection of C4 (18) and C2 fragments.

ACKNOWLEDGEMENTS

This study was supported by grants from Swedish Medical Resarch Council (B83-16X-68), the Medical Faculty, University of Lund, Konung Gustav V:S 80-års fond, Riksförbundet mot Reumatism, Greta och Johan Kochs stiftelser, Thorsten och Elsa Segerfalks stiftelse, Kungliga Fysiografiska Sällskapet i Lund, Alfred Österlunds Stiftelse, Lasarettet i Lund and Förenade liv, Ömsesidigt gruppförsäkringsbolag, Stockholm.

REFERENCES

1. Aarden L.A., deGrot E.R. & Feltkamp T.E.W.: Chrithidia luciliae a simple substrate for the determination of anti-dsDNA with immunofluorescence technique. Ann. N.Y. Acad. Sci. 254 : 505, 1975.

2. Abrass C.K., Nies K.M., Louie J.S., Border W.A. & Glassock R.J.: Correlation and predictive accuracy of circulating immune complexes with disease activity in patients with systemic lupus erythematosus. Arth.Rheum. 23 : 273, 1980.

3. Bourke B.E. , Moss I.K. & Maini R.N.: Measurement of the complement C3 breakdown product C3d by rocket immunoelectrophoresis. J. Immunol. Methods. 48 : 97, 1982.

4. Brandslund I., Sierstad H.C, Svehag H.C. & Teisner B.: Double-decker rocket immunoelectrophoresis for direct quantitation of complement C3 split products with C3d specificities in plasma. J. Immunol. Methods. 44 :63, 1981.

5. Cameron J.S., Lessof M.H., Ogg C.S., Williams B.D. & Williams D.G. : Disease activity in nephritis of systemic lupus erythematosus in relation to serum complement concentrations. DNA-binding capacitiy and precipitating anti-DNA antibody. Clin. Exp. Immunol. 25 : 418, 1976.

6. Cohen A.S., Reynolds W.E., Franklin C.E., Kulka E.C., Ropes M.W., Schulman L.E. & Wallace S.L. : Preliminary criteria for the classification of systemic lupus erythematosus. Bull. rheum. Dis. 21 : 643, 1971.

7. Cooper N.R., Nemerow G.R. & Mayes J.T.: Methods to detect and quantitate complement activation. Springer Semin. Immunopathol. 6 : 195, 1983.

8. Davis P., Cumming R.H. & Verrier-Jones J.: Relationship between

anti-DNA antibodies, complement consumption and circulating immune complexes in systemic lupus erythematosus. Clin. Exp. Immunol. 28 : 226, 1977.

9. Fiedler A.H.L., Walport M.J., Batchelor J.R., Rynes R.I., Black C.M., Dodi I.A. & Hughes G.R.V.: Family study of the major histocompatibility complex in patients with systemic lupus erythematosus: importance of null alleles of C4A and C4B in determining disease susceptibility. Brit. Med. J. 286 : 425, 1983.

10. Gewurz A., Lint T.F., Roberts J. L., Zeits H. & Gewurz H.: Homozygous C2 deficiancy with fulminant lupus erythematosus. Severe nephritis via the alternative complement pathway. Arth. Rheum. 21 : 28, 1978.

11. Johnson U. & Holmström E.: C3 fragmentation in human serum: formation of a mixed disulphide between C3d and albumin. Acta path. microbiol. immunol. scand. Sect. C 90 : 321 - 326, 1982.

12. Johnson U.: Influence of ageing and polyethyleneglycol on the quantitation of C3d/dg in plasma and serum. Acta path. microbiol. Immunol. scand. Sect C. In press..

13. Laurell A.-B., Mårtensson U. & Sjöholm A.G. : Studies on C1 subcomponents in chronic urticaria and angioedema. Int. Archs. Allergy appl. Immun. 54 : 434 , 1977.

14. Laurell A.-B., Mårtensson U. & Sjöholm A.G. : The development of simple tests for C1q, C1r, C1s, C2 and properdin. In Clinical aspects on the complement system, edited by W. Opferkuch, K. Rother, D.R. Schultz. Int. Symp., Bochum 1976, p 12 (Thieme, Stuttgart 1978).

15. Laurell A.-B., Mårtensson U. & Sjöholm A.G.: Quantitation of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ inactivator complexes by electroimmunoassay. Acta pathol. microbiol. Immunol. scand., Sect. C, Immunol. 87 : 79, 1979.

16. Lightfoot R.W. & Hughes G.R.V. : Significance of persisting serological abnormalities in SLE. Arth. Rheum. 19 : 837, 1976.

17. Lloyd W. & Schur P.: Immune complexes, complement and anti-DNA in exacerbations of systemic lupus erythematosus (SLE). Medicine 60 :208, 1981.

18. Milgrom H., Curd J.G., Kaplan R.A., Müller-Eberhard H.J. & Vaughan J.H.: Activation of the fourth component of complement (C4): assessment by rocket immunoelectrophoresis and correlation with the metabolism of C4. J. Immunol. 124 : 2780, 1980.

19. Morrow W.J.W., Williams D.J.P., Ferec C., Casburn-Budd R., Isenberg D.A., Paice E., Snaith M.L., Youinou P & Le Goff P.: The use of C3d as a means of monitoring clinical activity in systemic lupus erythematosus and rheumatoid arthritis. Ann. Rheum. Dis. 42 : 668, 1983.

20. Pangburn M.K., Schreiber R.D. & Müller-Eberhard H.J.: Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b like activities by spontaneous hydrolysis of the putative thioester in native C3. J. Exp. Med. 154 : 856, 1981.

21. Perrin L.H., Lambert P.H. & Miescher P.A.: Complement breakdown products in plasma from patients with systemic lupus erythematosus and patients with membranoproliferative or other glomerulonephritis. J. Clin. Invest. 56 : 165, 1975.

22. Petz L.D., Powers R., Fries J.R., Cooper N.R. & Holman H.R.: The in vivo metabolism of the third component of complement in systemic lupus erythematosus. Arth. Rheum. 20 : 1304, 1977.

23. Schur P. : Complement in lupus. Clin. rheum.Dis. 1 , 519, 1975.

24. Schutte M., DiCamelli R., Murphy P., Sadove M. & Gewurz H.: Effects of anestesia, surgery and inflammation upon host defence

mechanisms. I. Effects upon the complement system. Int. Archs. Allergy appl. Immun. 48 : 706, 1975.

25. Scullion M., Balint G. & Whaley K. : Evaluation of the C1q solidphase binding assay for immune complexes. A clinical and laboratory study. J. clin. lab. Immunol. 2 :15 , 1979.

26. Siegel S.: Nonparametric statistics for the behavioral sciences, Mc Graw Hill, New York, 1956.

27. Sjöholm A.G.: Complement components in normal serum and plasma quantitated by electroimmunoassay. Scand. J. Immunol. 4 ,25, 1975.

28. Sturfelt G., Sjöholm A.G. & Svensson B. : Complement components, C1 activation and disease activity in SLE. Int. Archs Allergy appl. Immun. 70 : 12, 1983.

29. Sturfelt G. & Sjöholm A.G.: Cleavage of C2 in pathological serum and plasma studied by crossed immunoelectrophoresis. Immunobiology (abstr) 164 : 302, 1983

30. Sturfelt G., & Sjöholm A.G.: Complement components, complement activation and the acute phase response in systemic lupus erythematosus (SLE). Int. Archs Allergy appl. Immun. In press.

31. Swaak A.J.G., Aarden L.A., Statius van Eps L.W. & Feltkamp T.E.W.: Anti-dsDNA and complement profiles as prognostic guides in systemic lupus erythematosus. Arth. Rheum. 22 : 226, 1979.

32. Tan E.M. & Kunkel H.G. : Characteristics of a soluble nuclear antigen precipitating with sera of patients with systemic lupus erythematosus. J. Immunol. 96 : 464, 1966.

33. Tan E.M., Cohen A.S., Fries J.F., Masi A.T., McShane D.J., Rothfield N.F., Schaller J.G.,Talal N. & Winchester R.J.: The 1982 revised criteria for the classification of systemic lupus erythematosus. Arth. rheum. 25 : 1271, 1982.

Journal of Immunological Methods, 63 (1983) 207–214
Elsevier

JIM 02761

Human Factor D of the Alternative Pathway: Purification and Quantitation by Enzyme Amplified Electroimmunoassay

Lennart Truedsson [1,*] and Gunnar Sturfelt [2]

[1] *Department of Immunology, Institute of Medical Microbiology, University of Lund, and* [2] *Department of Rheumatology, University Hospital of Lund, Lund, Sweden*

(Received 22 November 1982, accepted 21 April 1983)

Purified factor D was prepared with a yield of about 30%. Monospecific antiserum was raised in rabbits. Immunochemical quantitation of factor D in serum and plasma was performed by electroimmunoassay and a sensitive staining technique based on enzyme amplification. Peroxidase-labeled swine antibodies to rabbit immunoglobulin were applied to the gel after electrophoresis. Immune precipitates were then visualized by staining for peroxidase activity. Factor D could be detected at 50 μg/l. The normal concentration of D in serum was 1.6 mg/l, as assessed by this assay.

Key words: *complement — factor D — factor D purification — electroimmunoassay — immunoenzyme technique*

Introduction

Factor D (D) is a plasma protein playing an essential part in activation of the alternative complement (C) pathway (Müller-Eberhard and Götze, 1972; Hunsicker et al., 1973). It is a serine protease (Brade et al., 1974; Fearon et al., 1974) catalyzing cleavage of factor B (B) in the presence of C3b and Mg^{2+} (Lesavre et al., 1979), which results in the generation of the C3b, Bb complex with C3 cleaving activity.

Because of the low serum concentration of D, 1–2 mg/l (Lesavre and Müller-Eberhard, 1978; Lesavre et al., 1979), quantitation by ordinary immunochemical methods based on precipitation in gel is not possible. Functional assays have been described which use hemolysis of guinea pig erythrocytes (Martin et al., 1976), C3b-coated sheep erythrocytes (Lesavre and Müller-Eberhard, 1978) or rabbit erythrocytes (Lesavre et al., 1979). Measurement of D by radioimmunoassay has also been described (Lesavre and Müller-Eberhard, 1978).

* Address correspondence to: Dr. L. Truedsson, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

0022-1759/83/$03.00 © 1983 Elsevier Science Publishers B.V.

Löfberg and Grubb (1979) reported that, by introducing an enzyme amplifying technique, it was possible to increase the sensitivity of single radial immunodiffusion. In this study, we have found that this amplifying technique applied to electroimmunoassay results in an immunochemical method sensitive enough to be used in the determination of D in serum and plasma.

Materials and Methods

Normal serum and plasma

Blood specimens with 5 mmol/l EDTA, and samples without anticoagulant, were obtained from healthy blood donors. After centrifugation, serum and plasma were frozen at −80°C within 5 h of collection.

Reference serum

Sera from 148 normal blood donors were pooled and stored in aliquots at −80°C. This reference serum pool contained 100% D.

D-depleted serum (RD)

RD was obtained by gel filtration of fresh human serum on Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala) (Martin et al., 1976), or by passing fresh serum over a column of Bio-Rex 70 (Bio-Rad. Lab., Richmond, CA), as described by Lesavre et al. (1979).

Purification of D

D was purified from frozen human ACD plasma by a combination of ion-exchange chromatography and gel filtration as outlined by Volanakis et al. (1977). The fractions were tested for D by hemolytic assay, as originally described by Martin et al. (1976). Ultrafiltration on Amicon PM 10 membranes (Amicon Corp., Lexington, MA) was used to concentrate pooled D-containing fractions. The conductance of the buffers refers to values at 20°C.

Purification consisted of the following steps:

(1) After addition of 2 mmol/l EDTA, 3.5 l plasma was adjusted to pH 7.3 with 1 mol/l HCl and to a conductance of 11.0 mS/cm by dilution with distilled water. The plasma was then applied to a column of Bio-Rex 70 (7 cm × 23 cm) equilibrated with 50 mmol/l sodium phosphate, 2 mmol/l EDTA, pH 7.3 with a conductance adjusted to 11.0 mS/cm with NaCl (PBS). The column was washed, first with 1.5 l PBS containing 0.2% Triton X-100 (Eastman Kodak, Rochester, NY) (Lesavre et al., 1979) and then with 4.5 l PBS. The bound material was eluted with PBS adjusted to 30 mS/cm with a flow rate of 200 ml/h. As described by Tenner et al. (1981) D and C1q appeared in the same fractions under these conditions.

(2) The pooled fractions containing D (300 ml) were concentrated to 20 ml and applied to a column (4.5 cm × 90 cm) of Sepharose Cl-6B (Pharmacia Fine Chemicals, Uppsala) equilibrated with PBS, 40 mS/cm. The flow rate was 120 ml/h. The use of this gel made it possible to purify both D and C1q simultaneously.

(3) The D pool (400 ml), concentrated to 7.5 ml, was centrifuged to remove visible precipitates and the supernatant was filtered on a column (2.6 cm × 100 cm) of AcA 44 (LKB-Produkter AB, Bromma) equilibrated with 0.1 mol/l Tris (Sigma Chemicals, St. Louis, MO), 0.4 mol/l NaCl, 2 mmol/l EDTA, pH 7.5. The flow rate was 20 ml/h.

(4) The fractions containing D (95 ml) were dialyzed against 10 mmol/l barbital buffer, pH 7.4, adjusted to 15 mS/cm with NaCl (VBS). The dialysate was applied to a column of Bio-Rex 70 (2.3 cm × 6 cm) equilibrated with VBS and washed with 120 ml of this buffer. The bound material was eluted with the barbital buffer adjusted to 35 mS/cm. The flow rate was 60 ml/h, and 4 ml fractions were collected. Eight fractions with the highest D activity, comprising one-third of the fractions containing D, were pooled and concentrated to 2.6 ml. This preparation had a protein content of 0.55 g/l (or a total of 1.43 mg), as measured by the method of Lowry et al. (1951), with bovine serum albumin as reference protein.

Production of antiserum

The D preparation, 0.5 ml, was emulsified with 0.5 ml complete Freund's adjuvant (Difco Lab., Detroit, MI). The emulsion was injected subcutaneously at several points on the back of a rabbit. A booster injection was given 3 weeks later, and blood was collected after a further week, and every 2 weeks thereafter.

Other reagents

Rabbit anti-C5 antiserum was available in the laboratory.

Purified C1q was prepared by affinity chromatography as described by McKay (1981).

Enzyme amplified electroimmunoassay (EAE)

Electroimmunoassay was according to Laurell (1972), using 75 mmol/l barbital buffer containing either 2 mmol/l EDTA (VB-EDTA) or 2 mmol/l Ca^{2+} in the gel. After preliminary trials with agaroses having different endosmotic characteristics, a low endosmosis agarose, Agarose (LE) (FMC Corp., Marine Colloids Div., Rockland, ME) 1% (w/v) was found to be the most suitable. The gel was moulded on glass plates or preferably on agarose-coated polyester films (Gel Bond film; FMC Corp., Marine Colloids Div., Rockland, ME). To obtain distinct immunoprecipitates, 5% (w/v) polyethylene glycol (PEG) 6000 (Kebo AB, Stockholm) was added to the gel. Addition of PEG 20000 (Kebo AB, Stockholm) or Dextran T10 (Pharmacia Fine Chemicals, Uppsala) failed to improve the precipitation peaks. Antiserum to D, at a concentration of 0.07% (v/v), was added to the agarose. Five microliters of the samples in dilution 1/2 in VB-EDTA were introduced into 3 mm diameter wells in the agarose. Electrophoresis was carried out for 5 h at 20 V/cm. The gel was washed in 0.15 mol/l NaCl for 30 min, covered with a filter paper moistened with saline and a thick wad of soft absorbent paper on top, and pressed for about 10 min. The washing procedure was repeated twice.

The amplification technique described by Löfberg and Grubb (1979) for enzyme amplified single radial immunodiffusion was used to visualize the immunoprecipi-

tates. After washing, the gel was flooded with horseradish peroxidase-conjugated swine antibodies against rabbit immunoglobulins (Dakopatts, Copenhagen), diluted 1/20 in 50 mol/l phosphate buffered saline, pH 7.4, to which 5% non-immune swine serum was added. The gel was incubated with the labeled antibody in a moist chamber for 30 min. The washing procedure was then repeated 3 times. The immunoprecipitates were stained wine-red within 10–15 min on being immersed in a bath of 20 mg 3-amino-9-ethylcarbazole (Sigma Chemicals, St Louis, MO) dissolved in 2.5 ml dimethylformamide (BHH Chemicals, Poole), 50 ml 50 mmol/l acetate buffer, pH 5.0, and 25 μl hydrogen peroxide 30% (v/v) (Graham et al., 1965). After rinsing in water the gel was air-dried.

Immunochemical and electrophoretic methods

Double immunodiffusion in gel (Ouchterlony, 1967) was performed in a gel of the same composition as that used in the EAE, and the precipitates were visualized by the enzyme amplifying technique.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970).

Hemolysis in gel (HIG) assay for D

The hemolytic diffusion plate assay described by Martin et al. (1976) was used with minor modifications. Agarose plates containing guinea pig erythrocytes were prepared as described previously (Truedsson et al., 1981) with the addition of 5% (v/v) RD reagent. The buffer used was veronal buffered saline containing 5 mmol/l Mg^{2+} and 10 mmol/l EGTA (VBS-Mg^{2+}-EGTA) (Truedsson et al., 1981). Wells, 3 mm in diameter, were punched and 5 μl of the samples were introduced into each well. Incubation was carried out at 4°C for 16 h, and at 37°C for 2 h.

Results

Purified D

When analyzed by 12% SDS-PAGE the isolated protein gave only one band (Fig. 1), representing a molecular weight of approximately 25,000 daltons. When injected into a rabbit, the protein produced an antiserum which without absorption was monospecific for D. The plasma used for the preparation had a D concentration 78% of the reference serum (= 1.25 mg/l, see below) as determined by the EAE. Thus, the purification procedure resulted in 1.43 mg D from 3.5 l of the initial material, a yield of approximately 30%.

Antiserum specificity

Using unabsorbed anti-D antiserum, enzyme amplified double immunodiffusion gave rise to a single precipitation line, demonstrating a reaction of identity between D in normal human serum (NHS) and the purified D preparation (Fig. 2). Mixtures of equal volumes of the antiserum — heated at 56°C for 30 min — and NHS in serial dilutions, were tested by the HIG assay for D. Complete inhibition was

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the D preparation. Eleven micrograms of the preparation were applied to the gel, and the electrophoresis run under reducing conditions on a 12% gel. The anode was at the bottom.

Fig. 2. Enzyme amplified double immunodiffusion demonstrating an identity reaction between normal human serum (NHS) and purified D diluted 1:20 in VB-EDTA (D) obtained with anti-D antiserum diluted 1:4 in the same buffer (anti-D).

demonstrated with the anti-D antiserum in dilutions up to 1/16. Further dilution of the antiserum gave partial inhibition (Fig. 3A).

In a control experiment with antiserum to C5, another C component participating in the hemolytic reaction, the lytic pattern differed from that obtained with anti-D antiserum (Fig. 3B), indicating that inhibition by the anti-D antiserum is dependent upon specific binding of anti-D antibodies to D.

Enzyme amplified electroimmunoassay

Experiments with different buffers showed that in the presence of Ca^{2+} both

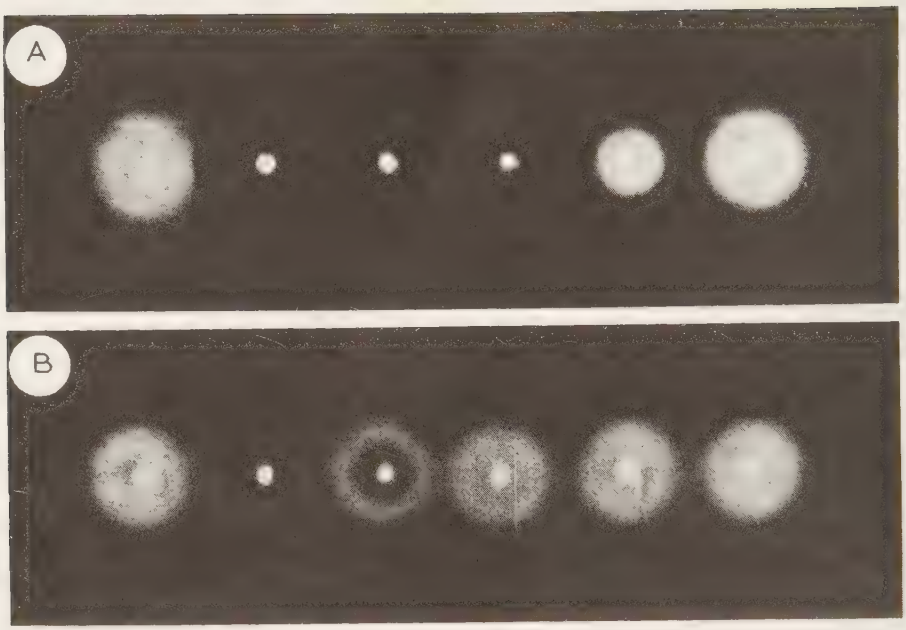


Fig. 3. A: influence of anti-D antiserum on D-activity in normal human serum (NHS) analyzed by the HIG assay for D. Reading from the left, the wells contain mixtures of: equal volumes of NHS and VBS-Mg^{2+}-EGTA, NHS and anti-D antiserum undiluted and in dilutions 1 : 4, 1 : 16, 1 : 64 and 1 : 256. B: results, obtained from a control experiment with anti-C5 antiserum, performed in the same way.

anodal and cathodal precipitation peaks were obtained with NHS. With EDTA in the buffer only anodal peaks were observed (Fig. 4). VB-EDTA was therefore used throughout the study. No precipitation peaks were found with RD.

Purified D diluted in VB-EDTA gave more sharply pointed anodal peaks than NHS. When D was diluted in RD, the resultant peaks were the same in appearance as those seen with NHS. It was also noted that higher peaks were obtained with purified D diluted in RD than with corresponding dilutions of D in VB-EDTA.

In the absence of PEG 6000 the peaks became blurred, making the heights of the rockets difficult to estimate. Cathodally located stained material was occasionally noted with NHS (Fig. 4) and RD. Purified C1q gave rise to cathodal precipitates with similar appearance. This was also seen in the absence of antiserum in the gel.

Measurable precipitation peaks were obtained with the reference serum in dilutions up to 1/32 in VB-EDTA, thus permitting detection of D concentrations as low as 3% of normal. The precision of the EAE was estimated by 20 duplicate determinations made on different occasions with sera of varying D concentrations. The standard deviation was calculated to be 9.5% (Weeke, 1973).

D in normal serum and plasma

The D content of the purified preparation was estimated to be 354-fold that of the reference serum by EAE analysis of dilutions of the preparation in RD. This

value was the mean of 5 separate determinations. Thus, on the basis of a protein concentration of 0.55 g/l in the D preparation, the D concentration in the normal serum standard was calculated to be 1.6 mg/l.

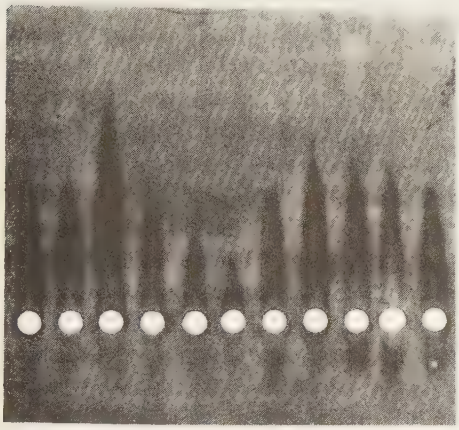


Fig. 4. Enzyme amplified electroimmunoassay of D in normal sera. From the left, precipitation peaks 3–6 represent the reference serum undiluted and in dilutions 1:2, 1:4 and 1:8.

D was quantitated in 20 normal human serum and plasma samples. The serum values varied between 65 and 140% (mean 102). Similar or somewhat lower values were obtained with corresponding plasma samples.

Discussion

The method of preparation resulted in highly purified D, as shown by analysis with SDS-PAGE. The yield was estimated as approximately 30%, which is higher than the values reported with previously described methods (Volanakis et al., 1977, Davis et al., 1979; Lesavre et al., 1979).

The introduction of the enzyme amplifying technique increases the sensitivity of the electroimmunoassay (Laurell, 1972) approximately 100-fold, and makes quantitation of D in serum possible. Low concentrations of D, approximately 50 μg/l can be determined. The amplifying step also made it possible to use very low concentrations of antiserum in the gel, thus economizing with an expensive reagent. This modification of the electroimmunoassay is therefore suitable for determining serum proteins present in concentrations too low to be measured by ordinary immunochemical assays based on precipitation in gels.

By use of the EAE, the concentration of D in NHS was determined as 1.6 mg/l, which agrees with values reported by others (Lesavre and Müller-Eberhard, 1978; Lesavre et al., 1979).

It was possible to obtain distinct anodal precipitation peaks in the presence of

EDTA, by addition of PEG 6000 to the gel. The occasional appearance of cathodally located stained material was not caused by D-anti-D precipitates, since this phenomenon was seen also in the absence of antiserum.

It has been reported that under certain conditions C1q interacts with agarose gels resulting in the formation of precipitates (McKay, 1981) and when purified C1q was tested with the EAE, cathodally located stained precipitates were indeed obtained. Low dilutions of serum, as well as the presence of PEG 6000 in the gel, may favour such 'nonspecific' protein-agarose interaction. Binding of labeled swine antibodies to C1q is a possible mechanism for staining these precipitates.

The anodal and cathodal precipitation peaks obtained with NHS in the presence of Ca^{2+} confirms the results reported by Davis et al. (1979). They found that, after agarose gel electrophoresis, purified D, as well as D in NHS, were distributed on both the cathodal and anodal sides of the application site with the highest D-activity towards the anode. It was reported by Konno et al. (1978) that purified D has β-mobility, whereas D in NHS has α-mobility in the presence of EDTA. The binding of D to another serum protein in the absence of Ca^{2+} and Mg^{2+} has been proposed (Konno et al., 1978), and such complex formation might explain why precipitation peaks of purified D diluted in RD differed from those obtained with D diluted only in buffer.

Acknowledgements

This work was supported by grants from the Swedish Medical Research Council (B83-16X-68-19C), Greta och Johan Kocks Stiftelser, Konung Gustav V:s 80-årsfond, Riksförbundet mot reumatism and Alfred Österlunds Stiftelse.

References

Brade, V., A. Nicholson, D. Bitter-Suermann and U. Hadding, 1974, J. Immunol. 113, 1735.
Davis III, A.E., C. Zalut, F.S. Rosen and C.A. Alper, 1979, Biochemistry 18, 5082.
Fearon, D.T., K.F. Austen and S. Ruddy, 1974, J. Exp. Med. 139, 355.
Graham, Jr., R.C., U. Lundholm and M.J. Karnovsky, 1965, J. Histochem. Cytochem. 13, 150.
Hunsicker, L.G., S. Ruddy and K.F. Austen, 1973, J. Immunol. 110, 128.
Konno, T., Y. Katsuno and H. Hirai, 1978, J. Immunol. Methods 21, 325.
Laemmli, U.K., 1970, Nature (London) 227, 680.
Laurell, C.-B., 1972, Scand. J. Clin. Lab. Invest. 29 (Suppl. 124), 21.
Lesavre, P.H. and H.J. Müller-Eberhard, 1978, J. Exp. Med. 148, 1498.
Lesavre, P.H., T.E. Hugli, A.F. Esser and H.J. Müller-Eberhard, 1979, J. Immunol. 123, 529.
Löfberg, H. and A.O. Grubb, 1979, Scand. J. Clin. Lab. Invest. 39, 619.
Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, J. Biol. Chem. 193, 265.
McKay, E.J., 1981, Immunol. Lett. 3, 303.
Martin, A., P.J. Lachmann, L. Halbwachs and M.J. Hobart, 1976, Immunochemistry 13, 317.
Müller-Eberhard, H.J. and O. Götze, 1972, J. Exp. Med. 135, 1003.
Ouchterlony, Ö., 1967, in: Handbook of Experimental Immunology, ed. D.M. Weir (Blackwell Scientific Publications, Oxford) p. 655.
Tenner, A.J., P.H. Lesavre and N.R. Cooper, 1981, J. Immunol. 127, 648.
Truedsson, L., A.G. Sjöholm and A.-B. Laurell, 1981, Acta Pathol. Microbiol. Scand. Sect. C 89, 161.
Volanakis, J.E., R.E. Schrohenloher and R.M. Stroud, 1977, J. Immunol. 119, 337.
Weeke, B., 1973, Scand J. Immunol. 2 (Suppl. 1), 37.

Acta path. microbiol. immunol. scand. Sect. C, 91: 383–389, 1983.

COMPLEMENT FACTOR D CONCENTRATIONS IN NORMAL SERA: COMPARISON OF IMMUNOCHEMICAL AND FUNCTIONAL DETERMINATIONS

[1]G. STURFELT and [2]L. TRUEDSSON

[1]Department of Rheumatology and [2]Department of Immunology, Institute of Medical Microbiology, University of Lund, Sweden

Sturfelt, G. & Truedsson, L.: Complement factor D concentrations in normal sera comparison of immunochemical and functional determinations. Acta path. microbiol. immunol. scand Sect. C, 91 383–389, 1983.

The concentration of factor D (D) was determined in the sera of 144 healthy adults and 29 healthy children by enzyme amplified electroimmunoassay (EAE), and by a hemolysis in gel (HIG) assay for D. The 95% geometric confidence areas for D in adults were 75–143% as determined by EAE, and 65–171% by the functional assay. The concentrations were given in per cent of a reference serum pool containing 1.6 mg/l of D as measured by EAE. The correlation coefficient between D values obtained by the two methods was $r = 0.52$ ($p < 0.001$). D levels were lower in children than in adults. In the EAE purified D, and pathological sera with very high D concentrations, gave lower precipitation peaks with dilutions performed in buffer than with dilutions in D depleted serum or human serum albumin

Key words: Complement; factor D; electroimmunoassay; immunoenzyme techniques; hemolytic assays

G. Sturfelt, Department of Rheumatology, University Hospital, S-221 85 Lund, Sweden.

Received 7.vi.83 Accepted 4.vii.83

Factor D (D) of the alternative complement pathway is a protease with a molecular weight of 23,500 (*Fearon et al.* 1974) present in serum at low concentrations, 1–2 mg/l (*Lesavre & Müller-Eberhard 1978; Lesavre et al.* 1979; *Truedsson & Sturfelt* 1983). Previously D has usually been determined by functional assays (*Martin et al.* 1976; *Wilson et al.* 1979). By employing a sensitive staining technique based on enzyme amplification, an adequately sensitive electroimmunoassay was developed (*Truedsson & Sturfelt* 1983). In the present study, D was measured by this enzyme amplified electroimmunoassay (EAE) in normal adults and in children. The results were compared with D values obtained using functional assays.

MATERIALS AND METHODS

Normal sera and plasma. Blood samples were collected from 144 registered blood donors, 112 men and 32 women, aged 19–61 (median 40 years), 6 healthy subjects aged 60–80 and from 29 healthy children aged 2–10 years (median 6 years). After centrifugation serum and EDTA-plasma were frozen in aliquots at −80 °C within 5 hours of collection.

Reference serum. A reference serum was obtained by pooling sera from 148 normal blood donors. D values were given in per cent of this reference serum containing D at 1.6 mg/l (*Truedsson & Sturfelt* 1983). The reference serum was stored in aliquots at −80 °C.

Purification of D. D was purified from human ACD plasma as described earlier (*Truedsson & Sturfelt* 1983).

Anti-D antiserum. A monospecific antiserum was obtained by immunizing rabbits with purified D emulsified in an equal volume of Freund's complete adjuvant (Difco Lab., Detroit, Mich., USA) (*Truedsson & Sturfelt* 1983).

Immune absorbent for D. The IgG fraction of anti-D antiserum was prepared by precipitation with 33% ammonium sulfate, followed by chromatography on DEAE cellulose (DE 32) (W. & R. Balston Ltd., Maidstone, England). The antibodies were coupled to cyanogenbromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) at 10 mg protein/ml gel, as recommended by the manufacturer.

D depleted serum (RD). A serum reagent depleted of D (RD) was obtained by the method described by *Lesavre et al.* (1979) or in some experiments by passing fresh norm-

TABLE 1. *Arithmetric Mean, Standard Deviation, Geometric Mean and 95% Geometric Reference Area for D in Serum Are Given. The Values Are Expressed in per Cent of the Reference*

	Arith. mean	Stand. dev.	Geom. mean	95% geom. reference area
Healthy adults (19–61 years, n = 144)				
EAE	105	17	104	75–143
HIG	109	25	106	65–171
Healthy children (2–10 years, n = 29)				
EAE	73	9	73	56–95
HIG	70	19	67	41–112

al serum through a column with the D immune adsorbent.

The RD gave neither lysis in the hemolysis in gel (HIG) assay for D (*Martin et al.* 1976), nor a precipitate in the EAE (*Truedsson & Sturfelt* 1983). The RD gave full lysis in a HIG assay for the classical pathway, but no lysis in a HIG test for the alternative pathway (*Truedsson et al.* 1981). Addition of highly purified D to RD at a concentration corresponding to that of D in normal serum, completely restored the alternative pathway function, as assessed by the HIG test.

Other reagents. Human serum albumin (HSA) was purchased from Sigma Chemicals, St. Louis, Mo., USA.

Enzyme amplified electroimmunoassay. A detailed

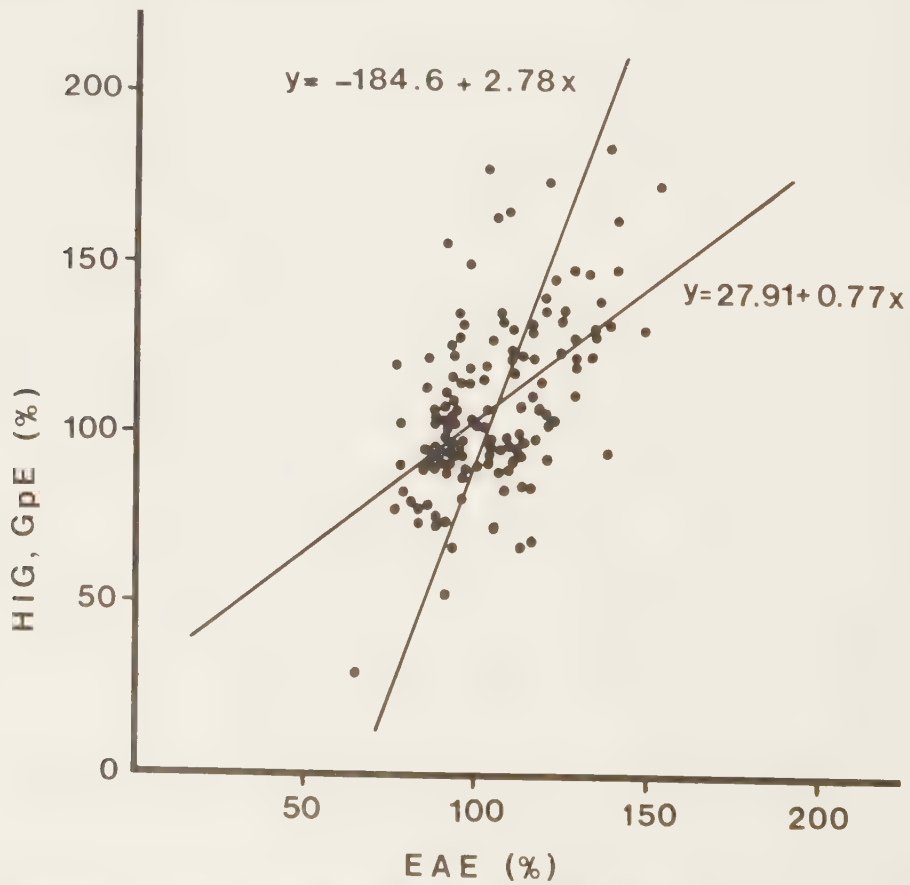


Fig. 1. D measured by EAE and HIG assay in sera from 144 normal subjects. Values are given in per cent of the reference.

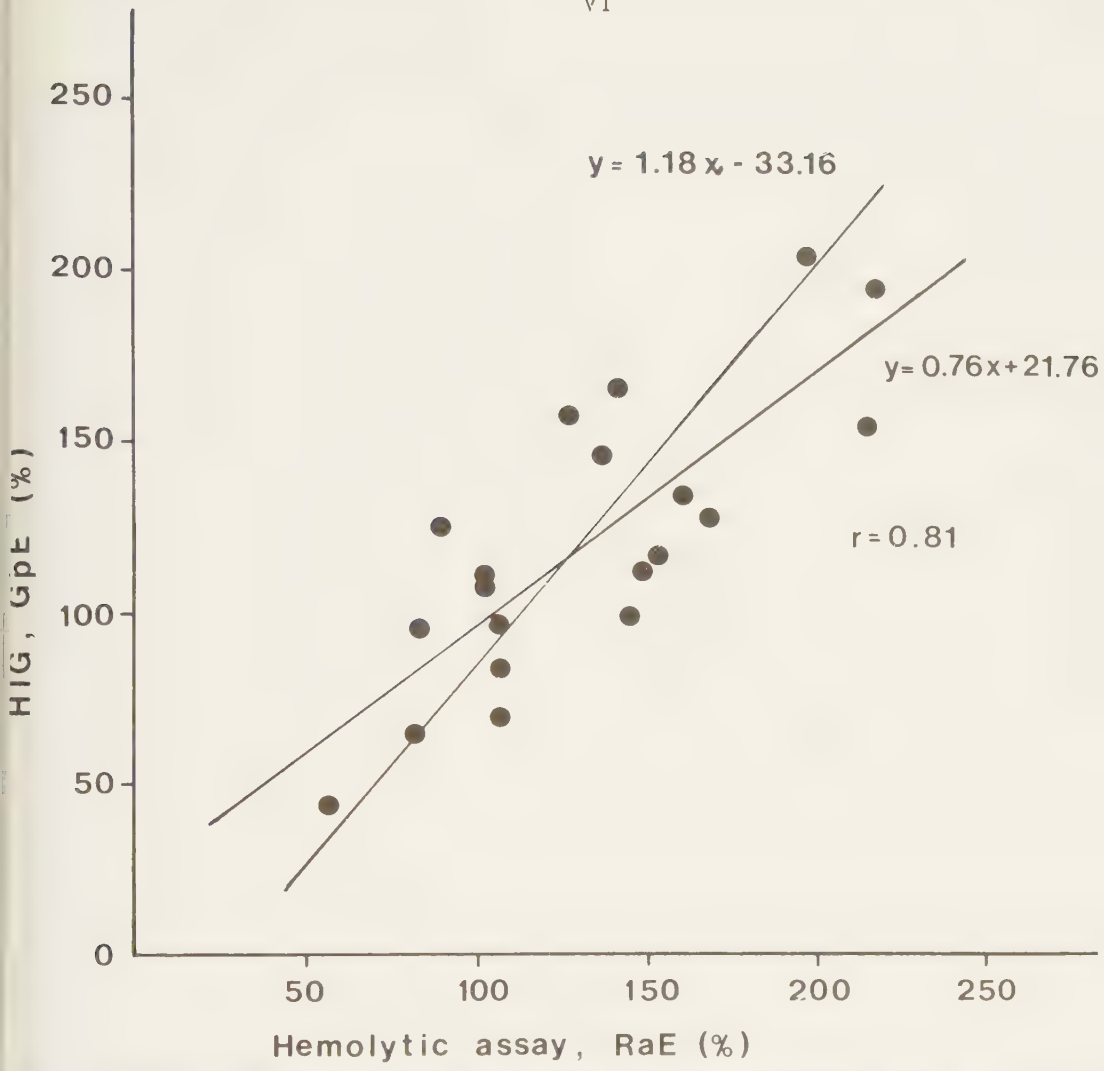


Fig. 2. Comparison of D activity measured by two hemolytic assays: HIG using guinea pig erythrocytes (HIG, GpE) and an assay using rabbit erythrocytes (RaE). Sera from 20 healthy subjects were studied. Values are given in per cent of the reference.

description of the method has been given (*Truedsson & Sturfelt* 1983). In short, the assay was performed with low endosmotic agarose, agarose (LE) (FMC Corp., Marine Colloids div., Maine, USA), using 75 mmol/l barbital buffer containing EDTA at 2 mmol/l (VB-EDTA). Polyethylene glycol 6000 was added to the gel at 5% (w/v), and anti-D antiserum at 0.07% (v/v). Electrophoresis was carried out for 5 h at 20 V/cm. After repeated washing in 0.15 mol/l NaCl, the gel was incubated for 30 min with peroxidase labelled anti-rabbit immunoglobulin. After further washing the immunoprecipitates were then stained by adding the substrate of peroxidase – viz., 3-amino-9-ethylcarbazole (Sigma Chemicals, St. Louis, Mo., USA).

Functional assays for D. A HIG assay for D was performed essentially as described by *Martin et al.* (1976); minor modifications were done as earlier described (*Truedsson & Sturfelt* 1983). The sera tested were applied diluted 1/2 in veronal-buffered saline with 5 mmol/l Mg^{2+} and 10 mmol/l EGTA (VBS-Mg^{2+}-EGTA).

D activity was also estimated by the tube assay described by *Lesavre et al.* (1979), based on hemolysis of rabbit erythrocytes.

The D activity present in test samples was expressed in per cent of that in the reference serum.

Statistical evaluation. Because of the positively skewed distribution of the values for D concentration, geometric means and 95% geometric reference areas were given.

Correlations and regression lines were calculated by the method of least squares. Logarithmic transformation of data was performed for calculation of age and sex differences. Significance of differences between paired data was calculated using Student's t-test, and reproducibility according to *Weeke* (1973).

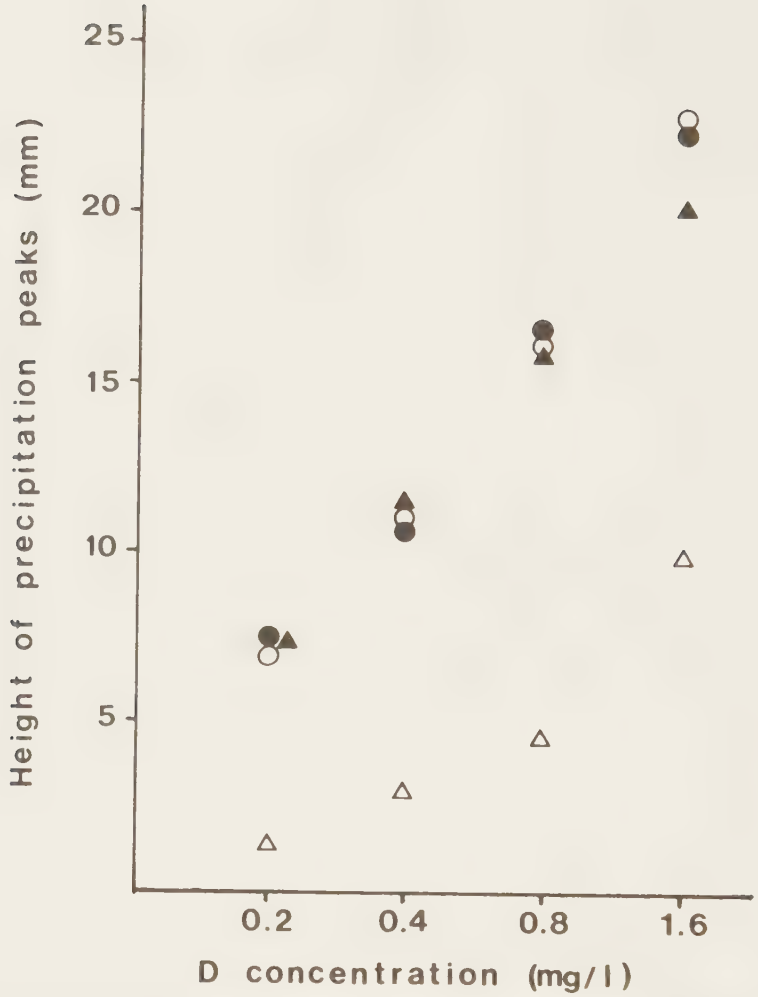


Fig 3a Measurement of D protein by EAE. NHS (circles) and purified D (triangles) were diluted in VB-EDTA (open symbols) and RD (filled symbols).

RESULTS

D Concentrations in Normal Sera

D levels were determined in 144 normal sera by the EAE and by the HIG assay for D. The results are given in Table 1. Among the sera studied the geometric mean concentration of D was compared in males and females. In males the mean concentrations of D was 108% by HIG and 105% by EAE and in females 98% in both assays. The difference between the mean D values in the two groups was barely significant ($p<0.05$) when determinations were made with HIG while the difference between the EAE values was not significant.

To assess influence of age on D serum concentration, the mean D value of 72 normal blood donors aged 19–40 was compared to that of 72 blood donors aged 40–61 years. The geometric mean values for the two groups were 103 and 105 with EAE and 102 and 109 with HIG, respectively. These differences did not reach significance ($p>0.1$).

In the sera from 29 healthy children (Table 1), D values were lower than in sera from adults ($p<0.001$).

Sera from 6 elderly subjects showed D concentrations (range 80–157% with EAE and range 91–174% with HIG), which were within the range for the healthy blood donors.

Comparsion between Immunochemical and Functional Quantitation of D

Figure 1 shows the relationship between the values obtained by the two methods. The equations $y = -184.6 + 2.78\ x$ and $y = 27.91 + 0.77\ x$, where x denotes the EAE estimates, give the mathematical

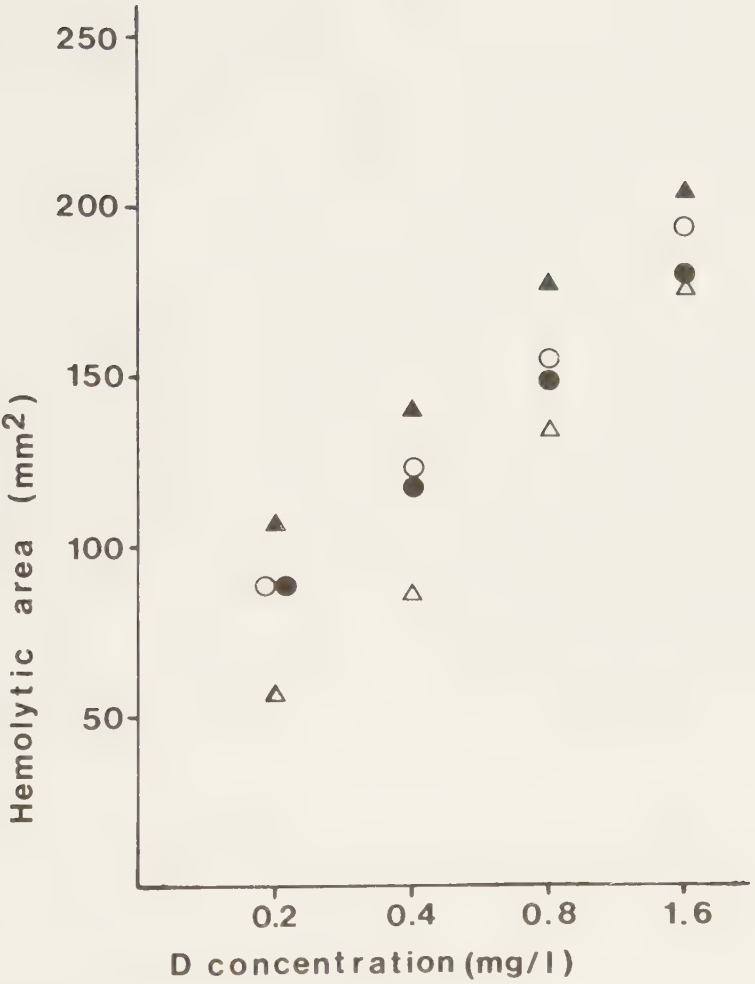


Fig. 3b. Measurement of D hemolytic activity by the HIG assay for D. The same symbols as in figure 3a are used.

relationship between D values in per cent of the reference. Functional and immunochemical values were correlated, $r = 0.52$ ($p < 0.001$). The differences (paired t-test) between the values obtained with the two methods were not statistically significant. In the high range, the functional assay gave higher values than the immunochemical method; and within the low range, functional D values tended to be lower than those determined immunochemically. These differences were not significant, however, nor was the difference between mean D values as estimated with the two methods.

Twenty normal sera were selected from high, low and mid-range areas, as determined by the EAE, for further comparison of functional and immunochemical methods for determinating D.

The two hemolytic assays gave similar values with a marked correlation ($r = 0.81$) (Fig. 2). The D values obtained by EAE showed the same relationship to the D activity found with the two hemolytic assays.

Duplicate determinations of the 20 sera were carried out to assess the reproducibility of the EAE and the HIG assay for D. The variation coefficient for the EAE was 9% and for the HIG assay 23%.

Influence of Diluents

Normal serum (NHS) and purified D at normal serum concentration (1.6 mg/l) were serially diluted in VB-EDTA and RD. Simultaneous estimations were performed with the EAE and with the HIG assay (Figs. 3a and 3b). Fairly linear dose-response curves were obtained with both assays.

When the D preparation was diluted in VB-EDTA, low and often sharply pointed immunoprecipitates were found, different from those obtained

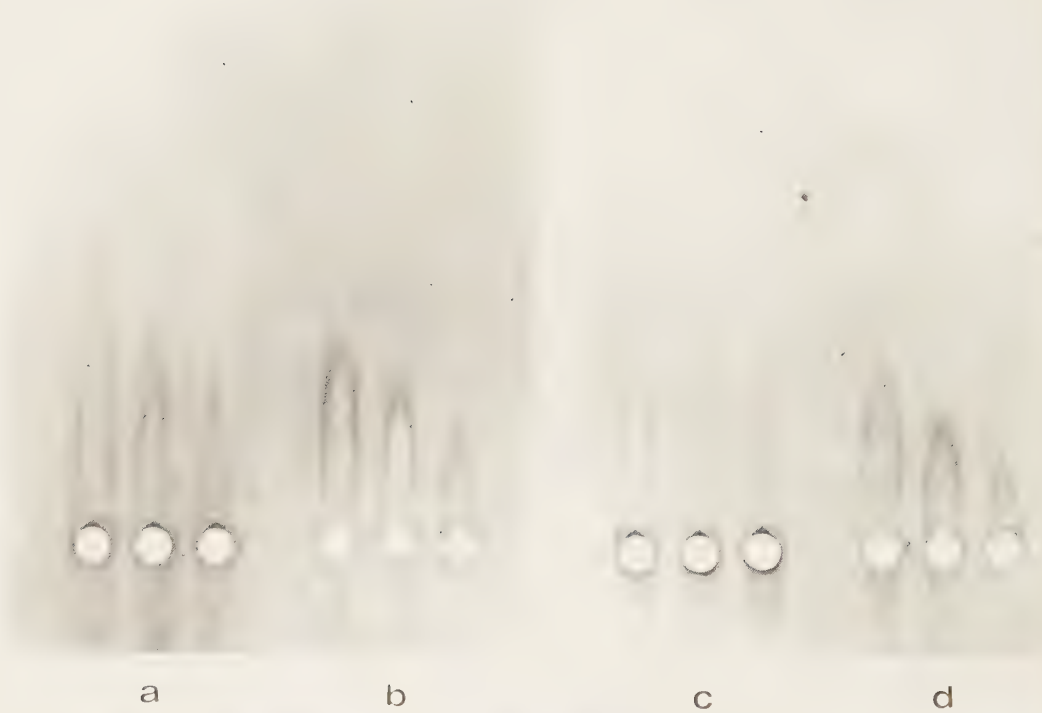


Fig. 4. Immunoprecipitates obtained by EAE with serum diluted in RD (a) and VB-EDTA (b) from a patient with high D level (800% of the reference). For comparison purified D was added to RD at 8-fold the normal serum concentration and then diluted in RD (c) and VB-EDTA (d). Reading from the left, the samples were tested at dilutions 1/8, 1/16 and 1/32.

with normal sera. However, purified D diluted in RD gave immunoprecipitates which were similar to those obtained with normal sera. EAE performed with purified D, in the presence of HSA (40–60 g/l) and with normal sera, gave immunoprecipitates that were almost indistinguishable.

When pathological sera with very high D levels were diluted in buffer the immunoprecipitates obtained were proportionately lower than those found with corresponding dilutions in RD (Fig. 4).

DISCUSSION

In this study D was quantitated in normal sera by an electroimmunoassay with high sensitivity made possible by enzyme amplification. The reproducibility of this immunochemical method was superior to that of the commonly used functional assay for D.

The normal range of D was given by immunochemical and functional measurements and agreed with that in a previous report by *Wilson et al.* (1979) in which a HIG assay was used. In the 29 sera from healthy children, lower D values were found than for adults, in contrast to the previous finding of increased D serum concentrations in newborns (*Johnson et al.* 1983). The present results indicate, however, that concentrations of D in adult sera are little influenced by age or sex.

In general, good agreement was found between the values obtained by the EAE and by the HIG assay. The differences between values obtained by the two methods were not statistically significant.

Comparsion of functional and immunochemical measurements revealed that, in the low range, D values were often higher as estimated by EAE than by two functional assays, and that they were lower in the high range. Similar discrepancies between the results of functional and immunochemical methods reported for C2 may be explained by the presence of nonfunctional C2 protein (*Gibson et al.* 1976) but it is not known whether nonfunctional forms of D protein exist in serum.

Serial dilution of NHS yields linear dose-response curves in both the EAE and the HIG assay. When

purified D was diluted in buffer, values obtained by the EAE were low compared with those found when the D preparation was diluted in RD. In sera with D within the normal range, dilution of sera in buffer is permissible. In pathological sera with high D levels, however, falsely low D values were obtained by EAE on extended dilution of the sera in buffer. Thus it might be advisable to dilute sera to be tested in suitable protein solutions. The present data indicate that RD, or possibly HSA can be used.

It has been established that in the alternative C3 convertase activation, D can be replaced by trypsin (*Brade et al.* 1974) and by kallikrein (*DiScipio* 1982). The presence of such proteases in pathological sera might result in falsely increased D values when a functional assay is used. Thus immunochemical determination may constitute a more specific means of determining D in clinical samples.

This work has been supported by grants from the *Swedish Medical Research Council* (B83-16X-68), the *Medical Faculty at the University of Lund, Konung Gustaf V's 80-årsfond, Riksförbundet mot Reumatism, Greta och Johan Kocks Stiftelser, Föreningen för Bistånd åt Vanföra i Skåne,* and *Alfred Österlunds Stiftelse.*

REFERENCES

Brade, V., Nicholson, D., Bitter-Suermann, D. & Hadding, U.: Formation of the C3-cleaving properdin enzyme on zymosan. J. Immunol. *113:* 1735–1743, 1974.

DiScipio, R. G.: The activation of the alternative pathway C3 convertase by human plasma kallikrein. Immunology *45:* 578–595, 1982.

Fearon, D. T., Austen, F. & Ruddy, S.: Properdin factor D: Characterization of its active site and isolation of the precursor form. J. Exp. Med. *139:* 355–366, 1974.

Gibson, D. J., Glass, D., Carpenter, C. B. & Schur, P. H.: Hereditary C2 deficiency: diagnosis and HLA gene complex associations. J. Immunol. *116:* 1065–1070, 1976.

Johnson, U., Truedsson, L. & Gustavii, B.: Complement components in 100 newborns and their mothers determined by electroimmunoassay. Acta path. microbiol. immunol. scand. Sect. C. *91:* 147–150, 1983.

Lesavre, P. H., Hugli, T. E., Esser, A. F. & Müller-Eberhard, H. J.: The alternative pathway C3/C5 convertase: chemical basis of factor B activation. J. Immunol. *123:* 529–534, 1979.

Lesavre, P. H. & Müller-Eberhard, H. J.: Mechanism of action of factor D of the alternative complement pathway. J. Exp. Med. *148:* 1498–1509, 1978.

Martin, A., Lachmann, P. J., Halbwachs, L. & Hobart, M. J.: Hemolytic diffusion plate assays for factors B and D of the alternative pathway of complement activation. Immunochemistry *13:* 317–324, 1976.

Truedsson, L., Sjöholm, A. G. & Laurell, A. B.: Screening for deficiencies in the classical and the alternative pathways of complement by hemolysis in gel. Acta path. microbiol. scand. Sect. C. *89:* 161–166, 1981.

Truedsson, L. & Sturfelt, G.: Human factor D of the alternative pathway: purification and the quantitation by enzyme amplified electroimmunoassay. In press. J. Immunol. Methods.

Weeke, B.: Rocket immunoelectrophoresis. Scand. J. Immunol. *2:* suppl. 1: 37–47, 1973.

Wilson, W. A., Thomas, E. J. & Sissons, J. G. P.: Complement activation in asymptomatic patients with sickle cell anemia. Clin. exp. Immunol. *36:* 130–139, 1979.

Preprint from Acta Med Scand

Serum Level of Complement Factor D in Systemic Lupus Erythematosus—an Indicator of Glomerular Filtration Rate

G. STURFELT, L. TRUEDSSON, H. THYSELL and L. BJÖRCK

From the Department of Rheumatology and Department of Immunology, Institute of Medical Microbiology, and the Department of Nephrology, University Hospital, Lund, Sweden

ABSTRACT. Sturfelt G, Truedsson L, Thysell H, Björck L. (Departments of Rheumatology and Immunology, Institute of Medical Microbiology, and Department of Nephrology, University Hospital, Lund, Sweden.) Serum level of complement factor D in systemic lupus erythematosus—an indicator of glomerular filtration rate. Acta Med Scand 1984.

Serum levels of complement factor D, a low molecular weight (LMW) protein, were high and inversely correlated with glomerular filtration rate (GFR) determined in 19 patients with lupus glomerulonephritis (LGN). Factor D was significantly closer correlated with S-creatinine than were two other LMW proteins, β_2-microglobulin and γ-trace in 22 LGN patients. Close correlations between each of the LMW proteins and S-creatinine were found in patients with a non-inflammatory disorder, polycystic kidney disease. Slightly increased β_2-microglobulin concentrations were found in 15 of 20 systemic lupus erythematosus (SLE) patients without renal involvement, while factor D and γ-trace showed normal values in most of these patients. The findings imply that serum concentrations of complement factor D in SLE are mainly determined by the GFR. *Key words: complement factor D, γ-trace, β_2-microglobulin, glomerular filtration, SLE, polycystic kidney disease.*

The renal handling of low molecular weight (LMW) proteins decides their concentration in body fluids (1). LMW proteins are normally eliminated from the circulation by glomerular filtration, and then absorbed and catabolized by the proximal tubular cells. Lesser amounts seem to be eliminated by peritubular uptake and catabolism in the tubular cells (2).

If the production of a LMW plasma protein is constant, and it is not catabolized by other routes than through the kidney, its concentration in plasma could be a good indicator of renal function. β_2-Microglobulin (β_2m) has been the subject of considerable study in these respects. The serum concentration of this LMW protein has been shown to be influenced not only by renal function but also by malignant and inflammatory processes (1), which limits its usefulness as an indicator of renal function.

High concentrations of complement factor D, a LMW (23.5 kD) serine protease of the alternative pathway (3), were found by functional assay in the sera from patients with systemic lupus erythematosus (SLE) (4). The highest factor D values were observed in patients with reduced glomerular filtration rate (GFR). These findings prompted us to examine closely the relationship between factor D levels and renal function in SLE. A new, perhaps more specific assay for factor D was used (5). Factor D was quantified in SLE patients with and without renal involvement and in patients with a non-inflammatory renal disorder, polycystic kidney disease (PCD). Two other LMW proteins, β_2m (11.8 kD) and γ-trace (13.3 kD), were also determined for comparison.

Abbreviations: β_2m = β_2-microglobulin, EDTA = ethylene diamine tetraacetic acid, GFR = glomerular filtration rate, LGN = lupus glomerulonephritis, LMW = low molecular weight, LNGN = SLE without renal involvement, PCD = polycystic kidney disease, SLE = systemic lupus erythematosus, VB-EDTA = veronal buffer containing EDTA at 2 mmol/l.

PATIENTS

Forty-two consecutive patients with SLE from the Department of Rheumatology, University Hospital, Lund, were included in the study. The diagnosis of SLE was based on the presence of a multisystemic disease and a positive antinuclear antibody test. At least four of the criteria proposed by the American Rheumatism Association (6) were present in all patients. Twenty-one consecutive patients with PCD served as a control group.

Lupus glomerulonephritis (LGN)

Twenty-two of the SLE patients (3 males and 19 females, aged 12–57 (median 33) years) had signs of renal involvement (decreased GFR, proteinuria, and hematuria and/or pathological urinary casts). Renal biopsy was performed in all but four of these patients. Light microscopy (Claus Brun, Department of Clinical Chemistry, Kommunehospitalet, Copenhagen) showed proliferative glomerulonephritis in 11, epimembranous in 3, membranoproliferative in 2 and unclassifiable glomerulonephritis in 2 patients.

Immunofluorescence analysis of the biopsies was performed in 11 of the 18 patients. All the biopsies analysed contained significant granular deposits in the glomeruli (Svend Larsen, Department of Pathology, Herlev Hospital, Copenhagen). C3 was found together with Ig in all 11 patients and C4 in 4. Serum C4 was decreased (<53% of normal) in 13 patients and C3 (<70% of normal) in 10. Both C3 and C4 were low in 8 patients. CrEDTA clearance, determined in 19 patients, varied between 29 and 106 (median 73) ml/min/1.73 m^2 S-creatinine was 50–689 (median 100) μmol/l in the 22 patients.

Based on clinical judgement, the disease activity was low in 16 patients. Six patients showed evidence of active disease with arthritis ($n=4$), lupus exanthema ($n=3$) and thrombocytopenia ($n=1$). As judged from urinalysis and repeated renal function estimations, the renal involvement was considered active in 3 patients. Four patients were not receiving therapy, 18 were on corticosteroids (prednisolone, 3–60 mg/day, median 9.4), 7 on azathioprine, 1 on cyclophosphamide and 1 on chloroquine.

SLE without renal involvement (LNGN)

The LNGN group comprised 1 male and 19 female patients aged 25–62 (median 40). At investigation they had not shown hematuria or pathological urinary casts or significant proteinuria (>0.5 g/24 h). Two patients had moderately active arthritis, two active lupus exanthema and 16 had a low active disease.

C4 was decreased in 5 patients, C3 in 5, and both C3 and C4 in 3 patients. S-creatinine was 36–81 (mean 64) μmol/l. Six patients were not on therapy, 9 were on corticosteroids (prednisolone 4.5–7.5 mg/day), 1 was on azathioprine and 9 were on chloroquine.

Polycystic kidney disease

Twenty-one consecutive patients with PCD (13 males and 8 females, aged 18–74, median 45) without signs of inflammatory or infectious disease were studied. Their S-creatinine varied between 80 and 1390 (median 170) μmol/l.

METHODS

Serum and plasma samples were stored in aliquots at −80°C until analysed.

Factor D was quantitated in serum by enzyme amplified electroimmunoassay as previously described (5). Test samples were diluted 1 : 2 in 75 mmol/l barbital buffer containing EDTA at 2 mmol/l (VB-EDTA). Samples with high factor D levels (>200%) were reinvestigated diluted in normal serum depleted of factor D and the concentrations were evaluated against the standard diluted in the same way (7). The 95% confidence interval for healthy adult males and females, expressed as the concentration in a normal plasma pool, was 75–143% (1.2–2.3 mg/l).

$\beta_2 m$ was quantitated by radioimmunoassay according to Plesner et al. (8). The reference area was 1.03–1.92 mg/l.

γ-trace was determined with an enzyme amplified single radial immunodiffusion method (9) (Anders Grubb, Department of Clinical Chemistry, General Hospital, Malmö, Sweden). The reference area was 0.97–1.83 mg/l.

Other methods. Complement components C3 and C4 were determined by electroimmunoassay (10), CrEDTA clearance according to Brøchner-Mortensen (11), and S-creatinine essentially according to Heinegård and Tideström (12).

Statistics. Correlations were calculated by the method of least squares. Differences between correlation coefficients were calculated according to Hotelling (13).

RESULTS

Serum concentrations of the three LMW proteins in the study groups are given in Table I. Of the SLE patients without renal involvement, 15 had slightly increased β_2m values, 4 had increased serum concentrations of γ-trace, and 3 patients had increased serum concentrations of both γ-trace and factor D. The highest factor D value in serum (216% = 3.6 mg/l) among the non-nephritic patients was found in a 69-year-old woman. She had a moderately decreased CrEDTA clearance (60 ml/min). In the patients without signs of renal disease, no correlation was found between S-creatinine and any of the LMW proteins.

Fewer patients in the LGN group had increased S-creatinine than increased serum concentrations of the LMW proteins. In contrast, a similar number of PCD patients had increased S-creatinine and LMW protein values (Table I).

In the LGN group, serum concentrations of the three LMW proteins showed a close relationship with S-creatinine. The correlation between S-creatinine and factor D values (Fig. 1, r=0.91) was significantly higher (p<0.001) than between S-creatinine and γ-trace (r=0.63) and also higher (p<0.01) than between S-creatinine and β_2m (r=0.76). The correlations between the three LMW proteins were less marked (r=0.64–0.69).

The inverted values of S-creatinine and of the three LMW proteins were positively correlated with the CrEDTA clearance (Fig. 2). The closest correlation was found between factor D and CrEDTA clearance (r=0.92, p<0.001), and between S-creatinine and CrEDTA clearance (r=0.83, p<0.001). The differences between correlation coefficients were not significant.

No relationship was found between serum concentrations of the three LMW proteins and age, sex, extra renal organ involvement or therapy in the SLE patients. Factor D, γ-trace and β_2m concentrations were unaffected by the moderate variations in disease activity noted in the SLE patients.

In the patients with PCD, close correlations were found between S-creatinine and serum concentrations of the three LMW proteins (Fig. 1, r=0.93–94, p<0.001). Concentrations of the LMW proteins were also closely correlated (r=0.89–0.94, p<0.001).

Table I. *Serum concentrations of factor D, β_2-microglobulin (β_2m), γ-trace and creatinine in 22 patients with lupus glomerulonephritis (LGN), 20 SLE patients without renal disease (LNGN) and 20 patients with polycystic kidney disease (PCD)*

	LGN			LNGN			PCD			Normal (95% confidence limits)
	Range	Median	High[a] (n)	Range	Median	High[a] (n)	Range	Median	High[a] (n)	
Factor D (% of reference)	96–968	217	15	78–216	3	3	98–1 208	358	18	75–143
β_2m (mg/l)	1.5–15.5	4.0	19	1.5–3.3	2.3	15	2.2–21.2	4.0	20	1.03–1.92
γ-trace (mg/l)	1.5–10.0	2.5	17	0.7–1.9	1.2	4	1.6–8.8	2.6	15	0.97–1.83
S-creatinine (μmol/l)	50–680	98	9[b]	36–81	65	0[b]	80–1 390	172	19[b]	60–115 (♂) 50–100 (♀)

[a] No. of patients with values higher than normal.
[b] The different normal ranges for men and women are taken into account.

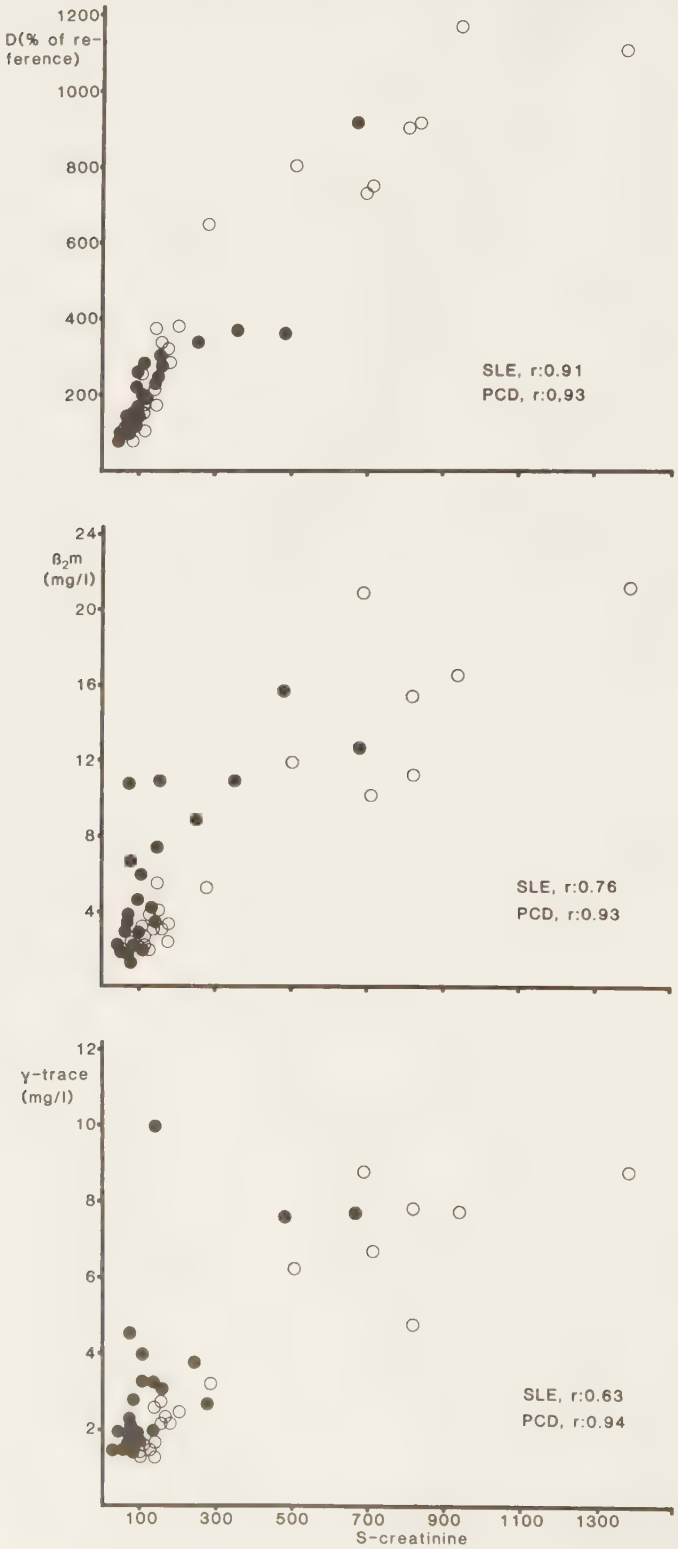


Fig. 1. Correlation between S-creatinine and complement factor D (*D*), β_2m and γ-trace in 22 SLE patients with glomerulonephritis (●) and 20 patients with polycystic kidney disease (○).

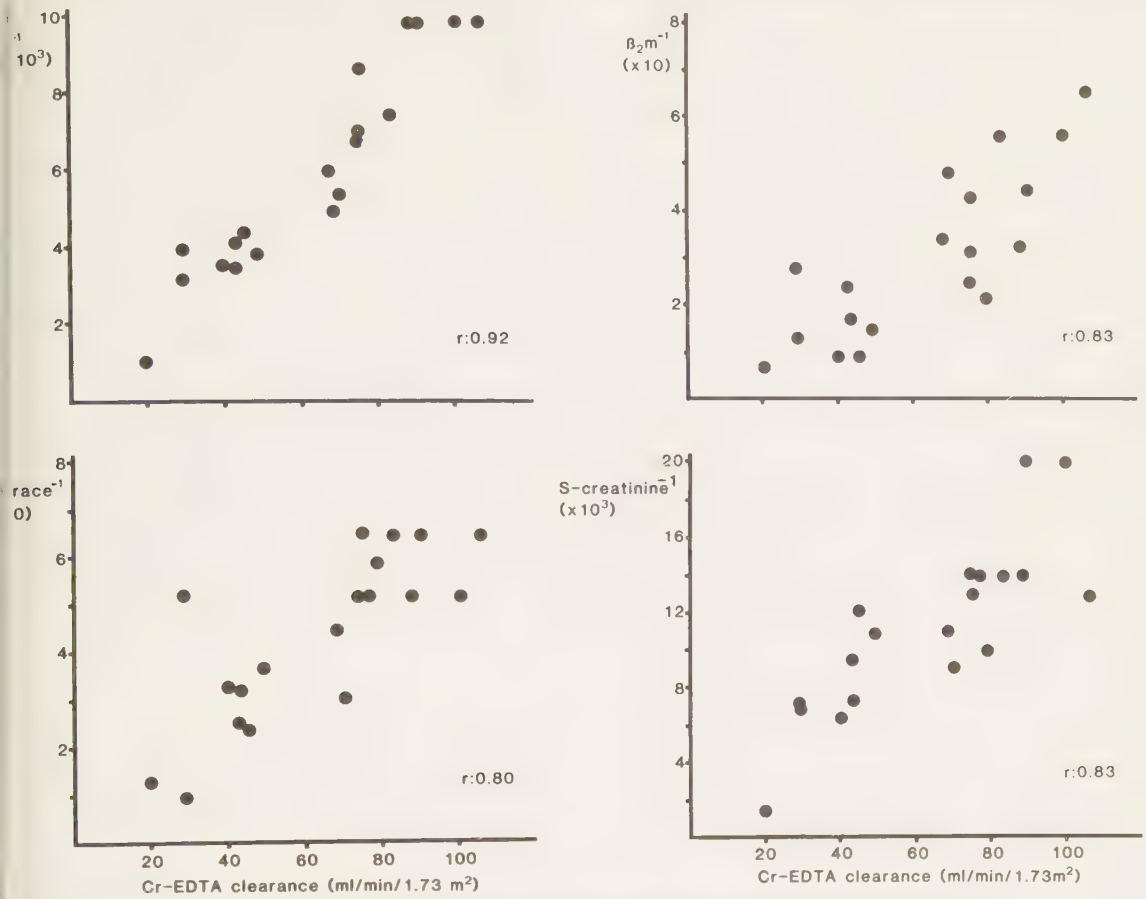


Fig. 2. Correlation between GFR as determined by CrEDTA clearance and the inverted values of factor D (*D*), β_2m and γ-trace in 19 SLE patients with renal involvement.

DISCUSSION

The previous findings of high serum concentrations of complement factor D in patients with severe SLE with renal involvement (4) were confirmed in the present study, and a close correlation was found between GFR and this LMW protein. S-creatinine was a less sensitive parameter for detection of reduced renal function in LGN patients than in the PCD group possibly related to low muscle mass in the former patients (14).

Factor D is present in the blood as an active enzyme and is not consumed in the course of complement activation (15). Factor D concentrations were normal in SLE patients with pronounced acute phase response and normal renal function (16). These observations support the importance of renal function as the main determinant of the serum concentrations of factor D.

Although the correlation of GFR with γ-trace and β_2m was numerically somewhat lower than with factor D in the present study, renal function should be of major importance in determining the serum concentrations of these two LMW proteins. Factors other than renal function appear to influence the serum concentrations of β_2m in rheumatic diseases (1, 17), which might explain why the correlation between GFR and β_2m was somewhat

less pronounced than between GFR and factor D. Since the biological role of γ-trace is unknown, no explanation of the differences between the serum concentrations of factor D and γ-trace is readily forthcoming.

As expected, the serum concentrations of factor D, β_2m and γ-trace were closely correlated with S-creatinine in patients with PCD. The production of the three LMW proteins seemed to be unaffected by increasing uremia, since the porportional increase in the serum concentrations of the LMW proteins was of the same order as that of S-creatinine. The present findings also indicate that peritubular uptake of these proteins is of minor importance since the tubular function is significantly disturbed earlier than the GFR in this disease (18).

The influence of age on the serum concentrations of β_2m (1) and γ-trace (19) has been pointed out. Slightly increased levels of factor D have been found in newborns (20). In adults the influence of age and sex on factor D concentrations in serum is slight (7).

In conclusion, the serum concentration of factor D, and possibly also of γ-trace, appeared to be a reliable indicator of GFR in patients with SLE nephritis. Owing to reduced muscle mass and increased tubular secretion of creatinine in such patients, GFR may often be overestimated when determined on the basis of S-creatinine concentrations (14, 21). The increased factor D concentrations in serum in one of our SLE patients without clinical signs of renal disease and with a normal S-creatinine but decreased GFR may illustrate the value of complement factor D as a sensitive indicator of reduced renal function.

ACKNOWLEDGMENTS

This investigation was supported by grants from the Swedish Medical Research Council (B83-16X-68), the Medical Faculty, University of Lund, Konung Gustaf V:s 80 års fond, Riksförbundet mot Reumatism, Greta och Johan Kocks Stiftelser, Thorsten och Elsa Segerfalks Stiftelse, Alfred Österlunds Stiftelse, Förenade Liv, Ömsesidigt Gruppförsäkringsbolag, Stockholm, Kungliga Fysiografiska Sällskapet i Lund, and Lasarettet i Lunds Fonder.

REFERENCES

1. Karlson FA, Wibell L, Ewrin P-E. β_2-microglobulin in clinical medicine. Scand J Clin Lab Invest 1980; 40 (Suppl 154): 27–37.
2. Ravnskov U, Johansson BG, Götlin J. Renal extraction of β_2-microglobulin. Scand J Clin Lab Invest 1972; 30: 71–5.
3. Fearon DT, Austen KF, Ruddy S. Properdin factor D. Characterization of its active site and isolation of the precursor form. J Exp Med 1974; 139: 355–66.
4. Sturfelt G, Sjöholm AG, Svensson B. Complement components, C1 activation and disease activity in SLE. Int Arch Allergy Appl Immunol 1983; 70: 12–8.
5. Truedsson L, Sturfelt G. Human factor D of the alternative pathway: Purification and quantitation by enzyme amplified electroimmunoassay. J Immunol Methods 1983; 63: 207–14.
6. Cohen AS, Reynolds WE, Franklin EC et al. Preliminary criteria for the classification of systemic lupus erythematosus. Bull Rheum Dis 1971; 21: 643–8.
7. Sturfelt G, Truedsson L. Complement factor D concentrations in normal sera: Comparison of immunochemical and functional determinations. Acta Pathol Microbiol Immunol Scand (Sect C) 1983; 91: 383–9.
8. Plesner T, Nörgaard-Pedersen B, Boenisch T. Radioimmunoassay of β_2-microglobulin. Scand J Clin Invest 1975; 35: 729–35.
9. Löfberg H, Grubb A. Quantitation of γ-trace in human biological fluids: indications for production in the central nervous system. Scand J Clin Lab Invest 1979; 39: 619–26.
10. Sjöholm AG. Complement components in normal serum and plasma quantitated by electroimmunoassay. Scand J Immunol 1975; 4: 25–30.
11. Brøchner-Mortensen J. A simple method for determination of glomerular filtration rate. Scand J Clin Lab Invest 1972; 30: 271–4.

12. Heinegård D, Tideström G. Determination of serum creatinine by a direct colorimetric method. Clin Chim Acta 1973; 43: 305–10.
13. Hotelling H. The selection of variates for use in prediction with some comments on the problems of nuisance parameters. Annals of Mathematical Statistics 1940; 11: 271–83.
14. Nived O, Sturfelt G, Westling H, White T. Is serum creatinine a reliable index of renal function in rheumatic diseases? Br Med J 1983; 286: 684–5.
15. Lesavre PH, Müller-Eberhard HJ. Mechanisms of action of factor D of the alternative complement pathway. J Exp Med 1978; 148: 1498–1509.
16. Sturfelt G, Sjöholm A. Complement components, complement activation and the acute phase reaction in SLE. Int Arch Allergy Appl Immunol. To be published.
17. Ström T, Ewrin P-A, Rehnberg E. Serum β_2-microglobulin (S-β_2) and β_2-microglobulin binding activity ($\beta_2\mu$ b.a.) in SLE. Scand J Rheumatol 1982; Suppl 45: 34 (abstract).
18. Martinez-Maldonado M, Yium JJ, Eknoyan G, Suki WN. Adult polycystic kidney disease: Studies of the defect in urine concentration. Kidney Int 1972; 2: 107–13.
19. Löfberg H, Grubb AO, Sveger T, Olsson J-E. The cerebrospinal fluid and plasma concentrations of γ-trace and β_2-microglobulin at various ages and in neurological disorders. J Neurol 1980; 223: 159–70.
20. Johnson U, Truedsson L, Gustavii B. Complement components in 100 newborns and their mothers determined by electroimmunoassay. Acta Pathol Microbiol Immunol (Sect C) 1983; 91: 147–50.
21. Hood B, Attman PO, Ahlmen J, Jagenburg R. Renal hemodynamics and limitations of creatinine clearance in determining filtration rate in glomerular disease. Scand J Urol Nephrol 1971; 5: 154–61.

Received Nov. 1, 1983.

Correspondence: G. Sturfelt, Department of Rheumatology, University Hospital, S-221 85 Lund, Sweden.